University of Alberta

Molecular and functional analysis of goldfish (*Carassius auratus* L.) macrophage growth factors

by

Patrick C. Hanington ©

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To granddad and grandpa.

Abstract

The innate immune responses of lower vertebrates, such as bony fishes, play a central role in host defence against infectious diseases. A competent immune response requires the continuous renewal of cells, and one of the most important effector cells of innate immunity are macrophages. Hematopoiesis is the process of mature blood cell formation and it is controlled by soluble factors known as cytokines. The developmental pathways initiated by hematopoietic cytokines are primarily driven by changes in expression of cell lineage-specific transcription factors and cell lineage-specific growth factors.

The central objective of my thesis research was to characterize and functionally analyze growth factors that regulate the development of macrophages in the goldfish. Three growth factors were identified, cloned and expressed and their function examined. Macrophage colony-stimulating factor -1 (CSF-1), granulin (Grn) and leukemia inhibitory factor (LIF) were each demonstrated to participate in the regulation of goldfish macrophage development.

The expression of CSF-1, granulin and LIF was examined using quantitative PCR and Northern blot analyses to determine the mRNA levels of each growth factor in the tissues and in specific sub-populations of cells involved in macrophage development. Using the transcript sequences of each growth factor, recombinant proteins were generated and used in functional assays that measured proliferation and differentiation of progenitor cells, monocytes and macrophages. These studies identified novel regulatory

mechanisms, distinct from those of mammals that control the development and function of macrophage of goldfish.

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List of abbreviations

A-SAA	Acute phase protein serum amylosis A
Ab	Antibody
AP	Alkaline phosphatase
AKT	Protein kinase B
AML	Acute myelogenous leukemia
AP-1	Activator protein-1
bp	Base pairs
BCA	Bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BLAST	Basic local alignment search tool
BR	Basic regions
BrdU	Bromodeoxyuridine
BS^3	Bis-sulfosuccinimidyl suberate
BSA	Bovine serum albumin
c-Cbl	Casitas B-lineage lymphoma
CBM	Cytokine binding motif
CCAC	Canadian council for animal care
CCL-71	Goldfish fibroblast cell line
CCM	Cell conditioned media
cDNA	Complementary deoxyribonucleic acid
cdk	Cyclin-dependent kinase
C/EBP	CCAAT/ Enhancer binding protein
CFU-G	Granulocyte colony forming units
CFU-GM	Granulocyte/monocyte colony forming units
CFU-M	Monocyte colony forming units
CH	Chromosome
CHO	Chinese hamster ovary
CMP	Common myeloid progenitor
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CpG	Cytosine and guanine separated by a phosphate
CSF-1	Macrophage colony stimulating factor
CSF-1R	Macrophage colony stimulating factor receptor
CT-1	Cardiotropin 1
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DIC	Differential interference contrast microscopy
DIG	Dioxigenin
Dlk	Delta-like 1 homolog
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
ECL	Electrochemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
Egr1,2	Early growth response 1,2
ELISA	Enzyme linked immune-sorbent assay
Epi	Epithelin
ERK	Extracellular regulated kinase
F	Forebrain
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FLT3	fms-related tyrosine kinase 3
Flk-2	Foetal liver kinase-2
gAD	Anteriodorsal ganglion
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS-1	Growth-arrest specific protein
GATA-1	Globin transcription factor-1
gAV	Anterioventral ganglion
G-CSF	Granulocyte colony stimulating factor
G-CSFR	Granulocyte colony stimulating factor receptor
GFP	Green fluorescent protein
GMP	Granulocyte-monocyte progenitor
Gp130	Glycoprotein 130
Grn	Granulin
gV	Trigeminal ganglion
gVII	Facial ganglion
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
hpf	Hours post fertilization
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
ICM	Intermediate cell mass
Ig	Immunoglobulin
IGFIIR	Insulin-like growth factor receptor II
IL-6	Interleukin six
IL-11	Interleukin eleven
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAK	Janus kinase
kb	kilobases
KC	Drosophila embryological cell line
K_d	Binding co-efficient

kDa	Kilodaltons
LB	Luria-Bertani broth
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LMP	Lymphoid-myeloid progenitor
LPS	Lipopolysaccharide
LZ	Leucine zipper
MAF	Macrophage activation factor
Man-6-P	Mannose-6-phosphate
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MEP	Megakaryocyte-erythroid progenitor
MITF	Microphthalmia-associated transcription factor
MO	Morpholino
M_r	Molecular weight
mRNA	Messenger ribonucleic acid
MSH	M17 homolog
NBT	Nitro blue tetrazolium chloride
NC	Notochord
NCBI	National Center for Biotechnology Information
N-CoR	Nuclear repressor co-repressor
NO	Nitric oxide
NOI	Nitric oxide intermediate
NTA	Nitrilotriacetic acid
nV	Trigeminal branchiomotor neuron
nVII	Facial branchiomotor neuron
nX	Vagal branchiomotor neuron
oligo	oligonucleotide
OSM	Oncostatin M
OSMR	Oncostatin M receptor
OV	Otic vessicle
PBI	Peripheral blood island
PBM	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween-20
PCDGF	PC-cell derived growth factor
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
Pgrn	Progranulin
PI-3	Phosphatidyl inositol-3
PIAS	Protein inhibitors of activated STAT

PKM	Primary kidney macrophage
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulphonyl fluoride
PNS	Peripheral nervous system
PTU	Phenylthiourea
RACE	Rapid amplification of cDNA ends
Rb	Retinoblastoma protein
RBI	Rostral blood island
rg	Recombinant goldfish-
RNA	Ribonucleic acid
RNAi	RNA interference / Interfering RNA
ROI	Reactive oxygen intermediate
RT-PCR	Reverse transcriptase polymerase chain reaction
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
sCSF-1R	Soluble macrophage colony stimulating factor receptor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9	Lepidopteran embryological cell line
SH2	*Src*-homology 2
SHC	*Src*-homology -2-domain containing
SHP-1	*Src*-homology -2-domain containing protein tyrosine phosphatase 1
SMRT	Silencing mediator for retinoid and thyroid-hormone receptors
SOCS	Suppressor of cytokine signalling 1
SSC	Sodium-chloride/Sodium citrate buffer
SSH	Suppressive subtractive hybridization
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TTBS	Tween-Tris-buffered saline
UTR	Un-translated region

Chapter 1

General Introduction

The production and renewal of blood cells for the maintenance of homeostasis and in response to various insults is under the rigid control of hematopoietic cytokines. These cytokines are functionally pleiotropic and their actions are mediated by an array of receptors that control cell survival, proliferation, differentiation, commitment, maturation and function. These cytokines can be specific to cell lineage or they can regulate the development of multiple cells lineages, and can act individually or concurrently to stimulate a specific response [1, 2]. Predominantly, hematopoietic cytokines act on basal hematopoiesis, which occurs, for the most part, in the primary hematopoietic organs. Several cytokines are also responsible for the initiation of "emergency" hematopoiesis initiated by various external as well as internal insults. Just as cytokines produced by effector cells are crucial for the regulation of an effective immune response in the face of challenge, hematopoietic cytokines are critical for the generation and maintenance of a pool of blood cells, as well as a rapid increase in specific cell types to tailor an immune response that contributes to the functional requirements of an organism [3].

Hematopoietic cytokines are produced by a variety of cell types and can act in paracrine, endocrine, juxtacrine or autocrine manner on their target cells. Thus, the hematopoietic cytokines can act in microenvironments and influence the renewal and development of specific blood cell lineages, and also act systemically on multipotent progenitor cells [4-6]. Cytokine sensitivity is determined by a complex regulatory network of activation and suppression signals. A hematopoietic cytokine may induce one cell developmental change in certain circumstances and a different developmental event when the circumstances are altered [2, 7]. Although specific cell lineages can be responsive to certain cytokines, there is a significant redundancy allowing for multi-level control of differentiation pathways [8-10].

The developmental events initiated by exposure to hematopoietic cytokines are primarily driven by changes in the expression of lineage-specific transcription factors [11]. Receptor signalling events result in downstream changes in the mRNA expression or protein conformation of transcription factors that bind to their complementary DNA enhancer and promoter regions inducing the expression of genes that affect the commitment of the target developing cell [12-15]. The developmental stage of a cell can commonly be determined most accurately not only by the expression of mRNA associated with cytokine receptors and cell surface markers, but also by the expression profile of transcription factors that control hematopoiesis [11, 16]. Sequence-specific DNA-binding factors also recruit co-factors to gene regulatory regions, many of which are part of multi-protein enzymatic complexes that facilitate or inhibit gene transcription by modification of chromatin [17, 18]. An important mechanism of chromatin modification is induced by retinoic acid that promotes the differentiation of myeloid cell lines, primary hematopoietic progenitors, and cells from patients with acute myelogenous leukemia (AML). The transcriptional activity of retinoic acid receptors can be modulated by a number of different growth factors, suggesting that in addition to the classical growth factor signalling pathways, certain growth factors can modulate stem cell fate via the retinoic acid pathway [19].

The primary body of work that contributed to the current state of knowledge regarding hematopoiesis was generated using the mouse model systems. The availability of hematopoietic cytokines, cell lines and knock-out mice has allowed researchers to map several hematopoietic pathways and functionally characterize a large number of molecules that play a role in hematopoiesis [20]. Although there is still much to be learned about mammalian hematopoiesis, knowledge generated using the mouse model systems has laid down a foundation for examination of hematopoiesis from an evolutionary perspective. With many of the cytokines and developmental markers defining hematopoietic cells or events already known for non-mammalian organisms, we can now begin to assess how hematopoiesis is regulated in lower organisms and whether and how it differs from that of mammals.

The study of hematopoiesis in non-mammalian organisms begins with the identification of the primary hematopoietic organs. In mammals, the bone marrow is the primary site of hematopoiesis for a majority of blood cells [21]. Since some non-mammalian organisms do not have bone marrow, the primary site of hematopoiesis, as well as the types of blood cell for the organism of interest, must be identified before more in-depth studies can be performed. One group of organisms in which hematopoiesis has been studied in detail are bony fishes (teleosts). Teleosts are the most ancestral vertebrate group to have well developed immune and hematopoietic systems that resemble mammalian systems [22]. The primary hematopoietic organ in the fish is the kidney, with the thymus and the spleen providing sites of more specific T-cell and macrophage/monocyte maturation, respectively [23, 24]. The repertoire of blood cells found in fish is similar to that of mammals; red blood cells are produced in conjunction with a multitude of progenitor and mature immune cells ranging from monocytes to NK-cells [23, 25-27].

Studies on the mechanisms of hematopoiesis in fish have primarily employed the zebrafish (*Danio rerio*) as a model organism. The strength of the zebrafish model system for examination of hematopoietic pathways is that it allows for monitoring of the transcriptional control of hematopoietically relevant genes and the knock-down systems (morpholinos) developed to assess their effect on cell development [28, 29]. Using zebrafish, a number of transcription factors have been identified in teleosts that control different stages of blood cell development. Perhaps one of the most interesting contributions that zebrafish studies have made to our understanding of hematopoiesis is the extensive characterization of the differences between primitive and definitive cell development [30-32].

Primitive and definitive hematopoieses occurs in vertebrates of all types, however, in teleosts it has been well established that both types are operational in adult fish and that they occur in different locations [33]. Primitive hematopoiesis in zebrafish induces the development of immune and blood cells until day 4 post fertilization. As the zebrafish mature, the intermediate cell mass (ICM), which is the area responsible for primitive hematopoiesis in the developing embryo, begins to migrate and forms a structure called the peripheral blood island (PBI) which finally resides along the spine in the posterior portion of the caudal vein [30, 34-37]. In addition to the PBI, primitive hematopoiesis has also been shown to carry over into adulthood in the rostral blood island (RBI) which appears to be primarily responsible for myeloid development during embryogenesis [33, 38]. The exact contribution of these sites of primitive hematopoiesis, to the repertoire of mature blood cells in the adult fish is unknown, however, analysis of the expression of transcription factors, and colony forming assays have shown that they remain active throughout the life of the fish [33, 34, 37]. Although studies done in zebrafish yielded significant results on the transcriptional regulation of stem cell development and cell commitment processes, functional studies using primary cells were difficult if not impossible to perform due to the size of zebrafish. For this reason, researchers have used larger fish (goldfish, carp) that belong to the same Order (Cypriniformes) and Family (Cyprinidae) as zebrafish, to further characterize the mechanisms of hematopoiesis in teleosts.

The goldfish/carp model system has allowed for functional analysis of hematopoiesis, due to their larger size and their ability to reproduce gynogenetically [39]. Researchers have identified a number of different cell types that arise from a population of progenitor cells found in the kidney. These progenitor cells have been shown to be able to differentiate into erythrocytes, lymphocytes, thrombocytes, granulocytes and monocytes [40-43]. Primary cultivation of the goldfish kidney cells has shown that they are capable of producing endogenous growth factors, and that addition of supernatants from previously grown cultures enhances the development of these cells *in vitro*. The ability of the non-transformed primary kidney cells to grow *in vitro* is finite, and following a period of rapid proliferation the cells enter a senescence phase characterized by cell clumping and programmed cell death [44, 45]. Analysis of the differentially expressed genes between proliferating and apoptotic cell cultures identified a number of growth and transcription factors that control cell proliferation. For example, goldfish granulin was shown to induce proliferation of the progenitor cell subpopulation [45, 46]. In addition to granulin, a number of hematopoietically relevant cytokines have been identified in the goldfish enhancing its usefulness as the fish model system for functional analysis of the mechanisms of hematopoiesis in teleosts. These include leukemia inhibitory factor (LIF) and LIF receptor [47, 48], macrophage colony stimulating factor (CSF-1) and CSF-1 receptor (membrane-bound and soluble receptor) [49, 50], the stem cell growth factor (SCF) and its receptor c-kit [45], granulocyte colony stimulating factor (G-CSF) and the G-CSF receptor, transforming growth factor beta (TGF-β) [51], epidermal growth factor (EGF), and platelet derived growth factor (PDGF). A growing list of developmentally relevant cytokines, receptors and cell surface markers that have been identified in the goldfish, and unique transplantation models that have been developed, allowing for detailed *in vivo* analysis of teleost hematopoiesis. For example, *in vitro* induction of gynogenesis in a goldfish/carp model system generated genetically identical individuals with different ploidy which allowed for passive transfer of primary kidney cells into anaemic (induced) or lethally irradiated goldfish. These studies confirmed that the kidney was the primary hematopoietic organ and suggested that the control of stem cell development and their commitment in the goldfish was similar to what is observed in mammals [39-42]. Initial functional studies suggested both similarities and divergence between mammalian and teleost hematopoiesis. In particular, teleosts employ unique regulatory mechanisms to control hematopoietic events [52]. Thus, the analysis of teleost hematopoiesis will undoubtedly shed new light on the development of the mechanisms of hematopoiesis from an evolutionary perspective.

Early studies of myelopoiesis in the goldfish in our laboratory [24, 43, 44, 49, 53, 54] described several features observed during the growth of primary kidney cells *in vitro*: (1) The *in vitro*-derived kidney monocytes/macrophages proliferated in culture for a finite period before reaching a senescence phase where proliferation of cells was reduced significantly and cells underwent apoptosis. Unlike mammalian monocytes/macrophages, goldfish monocytes/macrophages produced and secreted endogenous growth factors that were present in supernatants of the established cultures. The supplementation of the newly established primary kidney cell cultures with cell conditioned medium (CCM) from established cultures significantly enhanced the proliferation of the cells; (2) Goldfish kidney homogenates enriched for mononuclear cells characteristically consisted of small cells, which when analyzed by flow cytometry, were smaller than monocytes, macrophages or granulocytes, and had a simple internal complexity. The flow cytometric properties of the small primary kidney cells ("progenitor cells") changed during cultivation such that the initial cell pool developed into cell subpopulations that morphologically and cytochemically resembled monocytes and macrophages; and (3) In addition to the classical differentiation pathway, whereby semi-committed progenitor cells develop into blood monocytes and then into tissue macrophages upon encountering tissue microenvironments, it was observed that the sorted progenitor cell population was also capable of rapid differentiation and developed into mature fully functional macrophages. This 'alternative' differentiation pathway suggested that the progenitor cell population may contain cells that were responsive to different growth factors. It was suggested that the 'alternative' differentiation observed in goldfish primary kidney cell cultures was similar to the "embryonic" hematopoietic events observed in foetal mammals and "primitive" hematopoiesis in zebrafish. Since most hematopoietic events are induced after sequential exposure of the target cell to different growth factors, it was hypothesized that developmental events observed in the goldfish primary macrophage cultures were likely under the control of several growth factors.

1.1 Objectives of the Thesis

The central objective of my thesis was to characterize and functionally analyze growth factors that regulate myelopoiesis in the goldfish, with specific reference to: (1) the control of myeloid cell proliferation during development of macrophages *in vitro* and *in vivo*; (2) the regulation of cell commitment from uncommitted progenitor cells to functional and committed monocytes and macrophages; and (3) the control of progenitor cell development in the context of classical ("definitive") and alternative ("primitive") hematopoiesis.

The rationale for the analysis of specific growth factors in this thesis and their role in the molecular control of goldfish monocyte/macrophage development was based on previous studies done in our laboratory which reported that (A) differential cross screen analysis of mRNA transcripts yielded a number genes that encoded proteins known to be involved in control of cell proliferation and differentiation; (B) the cloning and characterization of the membrane bound and unique soluble form of the macrophage colony stimulating factor-1 (CSF-1) receptor which suggested the presence of the CSF-1 ligand in the goldfish. CSF-1 is one of the primary mammalian cytokines involved in monocyte/macrophage development that has been shown to regulate the commitment of myeloid progenitor cells, proliferation, and differentiation of mammalian macrophages; and (C) the presence of the IL-6 family of cytokines in teleosts, which are known to play an important role in mammalian hematopoiesis. One of the IL-6 family members identified in teleosts including goldfish was the LIF which has been shown to affect cell proliferation, development and activation of mammalian cells. One of the central functions of LIF in mammals is the maintenance of stem cell pluripotency. Identification of a putative teleost LIF in the goldfish [48] presented a unique opportunity to assess the evolutionary relationships and determine whether it was functionally similar to that of mammals

1.2 Outline of the Thesis

This document is the compilation of the work I have done for my PhD thesis that focused on the characterization of growth factors that control mononuclear cell development in the goldfish. Following this general introduction (Chapter 1), is the literature review chapter (Chapter 2) which summarizes current knowledge on myeloid cell development in mammals and fish. In Chapter 3, detailed materials and methods used during my PhD work are presented. Chapter 4, 5, 6, and 7 contain the description of and molecular and functional analyses of the three main growth factors that were studied: macrophage colony stimulating factor (CSF-1), granulin, and leukemia inhibitory factor. Chapter 8 is the general discussion and at the end of this chapter is the description of future directions that I believe should be undertaken to further characterize myeloid development in teleosts.

Chapter 2

Literature Review

2.1 Introduction

Macrophages are cells that are central components of the innate immune response and the maintenance of homeostasis. They are present in all tissues of an organism and play roles in a number of critical physiological processes such as: (a) the engulfment and removal of dead cells and cellular debris; (b) the production of more than 150 bioactive molecules that profoundly affect homeostasis; (c) the initiation of specific immune responses to pathogens by presenting antigens to T lymphocytes; and (d) the destruction of intracellular and extracellular pathogens and neoplastic cells.

The production of mature, differentiated myeloid cells (mononuclear cells and granulocytic cells) in mammals is regulated by the action of hematopoietic cytokines on progenitor cells in the bone marrow. Cytokines drive the process of myeloid differentiation by binding to specific cell-surface receptors in a stage- and lineage-specific manner. Following the binding of a cytokine to its cognate receptor, intracellular signal-transduction pathways become activated that facilitate the myeloid differentiation processes. These intracellular signalling pathways may promote myelopoiesis by stimulating expansion of a progenitor pool, supporting cellular survival during the differentiation processes, or by directly driving the phenotypic changes associated with differentiation.

2.2 Regulation of myelopoiesis

Development of mononuclear phagocytes occurs by a stepwise progression from pluripotent, uncommitted stem cells towards further committed cell stages until a final mature and functional effector cells is generated. Myelopoiesis which is a combined term for monopoiesis (development of macrophages) and granulopoiesis (development of granulocytes), begins with pluripotent hematopoietic stem cells (HSC) that give rise to a common myeloid progenitor (CMP), which in turn gives rise to a granulocyte-monocyte progenitor (GMP) [55, 56]. The GMP population includes granulocyte-, monocyte- and predominantly granulocyte/monocyte-colony forming units (CFU-G, CFU-M and CFU-GM). CFU-G and CFU-M likely arise from CFU-GM, though direct development of CFU-M or CFU-G from CMP or HSC under some circumstances also occurs. GMPs can also arise from a lymphoid-myeloid progenitor (LMP) [57, 58]. Foetal liver and adult bone marrow harbour cells with combined B-lineage and monocyte/macrophage potential [56, 59, 60], and the monocytes produced in the bone marrow not only develop into macrophages, but also give rise to osteoclasts and myeloid dendritic cells [61-63]. Regulation of the developmental commitment steps that direct myelopoiesis are largely under the control of growth factors (external sources) and transcription factors (internal sources). Growth factors exert their effects through the signalling events that occur once they bind to their corresponding receptor. These signalling events lead to changes in transcription factor expression patterns that control cell fate. Although many transcription factors have been linked to different stages of myelopoiesis in either a positive or negative regulatory role, the transcription factors belonging to the CCAAT/enhancer-binding proteins (C/EBPs), and PU.1 regulate the majority of myeloid genes in addition to activator protein-1(AP-1) proteins such as c-Fos and c-Jun, which activate at least a subset of monocytic genes [64-67].

2.3 Transcription Factors

The C/EBPs homo- and heterodimerize via their C-terminal leucine zipper (LZ) domains and bind DNA as dimers via the adjacent basic regions (BR) [68]. The co-crystal structure of the C/EBPα BR- leucine zipper (LZ) (bZIP) domain with its cognate binding site confirms that the bZIP domain is a continuous α-helix with residues 286–300 entering the major groove to make direct contact with the base pairs and the phosphate backbone of the DNA promoter region [69]. The consensus binding site is 50-T(T/G)NNGNAA(T/G)-30. C/EBPα, C/EBPβ and C/EBPδ have N-terminal trans-activation domains, and translation initiation from internal methionines produces truncated dominant-inhibitory polypeptides that retain the bZIP domain but have an altered range of preferred DNA-binding sites [70-73]. C/EBPα, C/EBPβ and C/EBPδ are predominantly expressed in the granulocyte, monocyte and eosinophil lineages [67, 74-77]. C/EBPα expression predominates in immature cells and is detected in the hematopoietic stem cell (HSC), common myeloid progenitor (CMP) and granulocyte/monocyte progenitor (GMP), but not the common lymphoid (CLP) or megakaryocyte-erythroid progenitor (MEP) populations [56], while C/EBPε is found in later-stage granulocytes [78] (Fig. 2.1).

Knockout studies using mice that are C/EBPβ (-/-) demonstrate that bone marrow cells generate 25–50% less myeloid colonies compared with C/EBPβ (+/-) cells in similar cytokine environments. The colonies formed are smaller, potentially reflecting an ability of C/EBPβ to stimulate the proliferation of myeloid cells, as observed with other cell types [79-81]. Induction of C/EBPβ *in vitro* may account for the ability of C/EBPα(-/-) cells cultured in IL-3 or GM-CSF, or transduced with the G-CSF receptor and cultured in G-CSF to generate neutrophils [80, 82]. In contrast to C/EBPβ, C/EBPα inhibits G1 to S cell cycle progression in a variety of cell lineages, including myeloid cells [83-85]. C/EBPα inhibits cell proliferation by several mechanisms that vary between cell lineages, including direct binding of E2F1 domain or cyclin-dependent kinase (cdk)2/cdk4 and induction of p21 [86]. Interaction of E2F1 with the outer surface of the C/EBPα BR enables growth inhibition of myeloid cells and is required for their terminal differentiation [87-89]. C/EBPα (-/-) neonatal mice lack neutrophils and eosinophils, although they retain monocytes in their peripheral blood [90]. Further, C/EBPα gene knockout studies suggest that a lack of C/EBPα results in a failed transition from a CMP to GMP; however these studies do not address the subsequent role of C/EBPα in monocyte versus granulocyte lineage commitment. The ability of exogenous C/EBPα and other C/EBPs to direct granulopoiesis from several myeloid cell lines resulted in early models of development that ascribe a more prominent role for C/EBPα during granulopoiesis than monopoiesis [67, 85, 91]. However, experiments on the transduction of C/EBPα into bone marrow mononuclear cells followed by lineage-depletion and then cultivation with or without estradiol, suggest that C/EBPα favours formation of monocytes over granulocytes in liquid culture. As well as CFU-M over CFU-G in methyl cellulose culture, in the presence of IL3/IL-6/SCF or GM-CSF [92]. Consistent with the conclusion that C/EBPα can direct monopoiesis and granulopoiesis, transduction of B- or T-cell progenitors with C/EBPα induces macrophage but not neutrophils development [93-95]; in addition, transplantation of mice with bone marrow transduced with C/EBPα increases the proportion of monocytes from 13% to 88% while inhibiting erythropoiesis [96]. In contrast, C/EBPα converts CD71$^+$ erythroid cells to granulocytes [97], likely via induction of the GATA binding protein 1 (GATA-1), which can directly bind and inhibit PU.1 [98] (Fig. 2.1).

The other important transcription factor that regulates myelopoiesis is PU.1. PU.1 binds as a monomer to the consensus DNA site 50-AAAG(A/C/G)GGAAG-30 via its C-terminal Ets domain and activates transcription via its N-terminal glutamine-rich and acidic domains [99]. PU.1 is expressed in B-lymphoid, early T-lymphoid, granulocytic and monocytic cells [99-102]. PU.1(-/-) mice lack B cells and monocytes, and have greatly reduced neutrophils [103-105]. These mice also have markedly diminished common lymphoid progenitors (CLP) and granulocyte-macrophage progenitors (GMP) and a characteristic increase in megakaryocyte-erythroid progenitors (MEP) [106, 107]. That PU.1 is essential for myeloid development is supported by the observation that expression of the macrophage colony stimulating factor receptor (CSF-1R) or granulocyte colony stimulating factor receptor (G-CSFR) in PU.1(-/-) bone marrow cells did not rescue myeloid development [108, 109]. The addition of low concentrations of recombinant PU.1 to PU.1(-/-) cell cultures induces granulopoiesis, whereas high concentrations of PU.1 are apparently required for induction of monopoiesis [110, 111] (Fig. 2.1).

Genetic analysis also indicates that a higher level of PU.1 favours monocytic over granulocytic development: lack of one PU.1 allele favours neutrophil development from embryonic stem cells *in vitro* and encourages neutrophil development *in vivo* in the absence of G-CSF [110, 112]. Deletion of the PU.1 distal enhancer located at -14 kb, results in a 80% decrease in PU.1 expression and subsequent loss of monopoiesis, while granulopoiesis remains unchanged [113]. The deletion of PU.1 in adult mice preserves the granulocyte cell pool and decreases the monocyte cell pool [114, 115]. Interestingly, these two studies also reported that the deletion of PU.1 causes a decrease in CSF-1R expression, with no effect on G-CSFR expression, suggesting that PU.1's effect on monopoiesis may be due to alterations in the expression levels of the CSF-1R. PU.1 is sufficient to reorganize the chromatin structure of the CSF-1R promoter in myeloid progenitors, but requires onset of early growth response 2 (Egr-2) expression to fully activate the intronic regulatory region [116, 117].

PU.1 binds and activates its own promoter and distal enhancer, potentially to variable degrees in different cell lineages and developmental stages [100, 118]. Onset of PU.1 expression in hematopoietic stem cells or lymphoid-myeloid progenitor cells is dependent on Runx1-mediated activation through the PU.1 distal enhancer [119]. C/EBPα then directs LMP or common myeloid progenitor (CMP) to the granulocyte-macrophage progenitor cell stage and beyond, in part through further PU.1 induction.

The C/EBPβ and PU.1 DNA-binding domains directly interact [120], and promoter-bound C/EBPβ increases PU.1 interaction with a nearby cis element in the IL-1β promoter, augmenting induction of various genes [121]. Similarly, C/EBPα also cooperates with PU.1 to regulate myelopoiesis [122]. Increased PU.1 levels in myeloid cells facilitates the ability of PU.1 to directly bind and repress trans-activation by GATA-1 to down regulate the erythroid development and up regulate myeloid development; GATA-1 inhibits PU.1 activity in cooperation with retinoblastoma protein (Rb) to ensure continued maturation of erythroid and megakaryocytic cells [123, 124]. In addition to favouring monopoiesis over granulopoiesis, high levels of PU.1 also favour myeloid over lymphoid development. Addition of exogenous recombinant PU.1 in PU.1(-/-) progenitor cell cultures induces B cell development at lower levels and increased development of monocytes when levels are increased [125]. Moreover, exogenous PU.1 converts B or T cell progenitors into monocyte/macrophages but not granulocytes [126, 127], and PU.1 knockdown in embryonic stem cell-derived CD34$^+$ cells favours B-cell formation [128]. The ability of both C/EBPs and PU.1 to reprogram lymphoid cells for monocyte lineage likely reflects the ability of C/EBPs to bind to and activate the PU.1 promoter and distal enhancer. These observations demonstrate the complex interplay among transcription factors involved in myeloid cell development (Fig. 2.1).

Activation and interaction with gene promoter and enhancer regions is not only controlled by transcription factors. Sequence-specific DNA-binding factors also recruit cofactors to gene regulatory regions, many of which are part of multi-protein enzymatic complexes which facilitate or inhibit gene transcription by modification of chromatin, the protein-bound state of DNA present in the cell [17, 18]. An important mechanism of chromatin modification is via retinoic acid, which promotes differentiation of myeloid cell lines, primary hematopoietic progenitors, and cells from patients with acute myelogenous leukemia (AML). In their unbound state retinoic acid receptors are bound by co-repressors such as nuclear repressor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid-hormone receptors (SMRT) [129]. Histone deacetylase (HDAC) activity associated with these complex results in local chromatin condensation and transcriptional silencing. Addition of retinoic acid induces an exchange of the co-repressor complex for transcriptional activators that possess histone acetyltransferase activity [130]. Activation of this process results in the remodelling of chromatin and the consequent transcriptional activation of target genes. HDAC can also recruit methyl-CpG binding proteins to occupy methylated promoter regions and thereby silence retinoic acid target genes [19, 130, 131]. Interestingly, the transcriptional activity of retinoic acid receptors can be modulated by a number of different growth factors, suggesting that in addition to the classical growth factor signalling pathways certain growth factors can also modulate stem cell fate by the retinoic acid pathway [19].

Along with PU.1 and C/EBPs, a number of other accessory transcription factors are involved in control of myelopoiesis. Although transcription factors and chromatin modifications control cell fate decisions, they also regulate the responsiveness of cells to different growth factors that control myeloid development [65].

2.4 Growth factors involved in myelopoiesis

Growth factor control of cell development is a complex web of positive and negative signals that depends as much on cell stage and type as the growth factors eliciting the effect. As indicated earlier, the important growth factors that influence myelopoiesis are the colony stimulating factors [CSF-1, G-CSF, granulocyte macrophage colony stimulating factor (GM-CSF) multi-CSF (IL-3) and stem cell factor (SCF)] [4]. However, in addition to these factors, a number of different cytokines can influence cell development; members of the IL-6 cytokine family, such as LIF and IL-6; pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and IL-1; other growth factors, such as granulin, epidermal growth factor (EGF) and transforming growth factor beta (TGF-β); and exogenous pathogen produced molecules, such as bacterial lipopolysaccharide (LPS) and peptidoglycan [1, 2].

Although multiple cytokines can influence myelopoiesis, there are a select few that drive myeloid developmental events under normal conditions. The colony-stimulating factors, CSF-1 (M-CSF), G-CSF, GM-CSF and multi-CSF or IL-3, all play critical roles in normal myeloid development. These factors show both functional pleiotrophy, exhibiting a wide variety of biological functions on various tissues and cells, as well as significant redundancy, being able to exert similar and overlapping functions on specific cells. Of the colony stimulating factors, CSF-1 and GM-CSF are of central importance for mononuclear cell development, whereas G-CSF drives granulocyte development, and IL-3 promotes the development of early myeloid, lymphoid and erythroid progenitors [4] (Fig. 2.2).

In this review I will discuss current knowledge of the growth factors I examined in my thesis: CSF-1 and its receptor, granulin, and LIF and its receptor.

2.4.1 Macrophage colony-stimulating factor (CSF-1)

Macrophage colony-stimulating factor is the principal regulator of the survival, proliferation, and differentiation of macrophages and their precursors [132, 133]. CSF-1 can also synergize with other cytokines to mediate the proliferation of early hematopoietic progenitors [134-136]. CSF-1 was originally identified in murine serum and human urine, and was later found to be a significant component of the cell-conditioned medium from the murine fibroblast L-929 cell line, which was used to supplement macrophage cultures *in vitro* [137-139]. In addition to being produced in a wide array of tissues, [139, 140] it has also been shown that CSF-1 is secreted by a number of cell types such as placental trophoblasts, fibroblasts and stromal cells which have been shown to be key producers of circulating CSF-1 [133, 141, 142]. CSF-1 production can also be induced through activation of various cell types including chondrocytes, monocytes, macrophages and T or B lymphocytes [4]. Importantly, the induction of CSF-1 production in monocytes and macrophages can be achieved after stimulation with a variety of cytokines, such as GM-CSF [143], TNF-α [144], IL-1 [145, 146], and IFN-γ [147, 148]. The capacity of monocytes/macrophages to produce CSF-1 suggests that these cells can auto-regulate their own proliferation and function to quickly respond in times of emergency. Interestingly, the CSF-1 mRNA expression in activated cells does not always correlate to the production of CSF-1 protein, implying a post-transcriptional regulation of CSF-1 translation or secretion [15].

Macrophage colony stimulating factor is encoded by a single gene that can undergo alternative splicing to generate three functional CSF-1 molecules ranging in size from 1.5 to 4.4 kb [10, 132, 149, 150]. Alternative splicing of CSF-1 can occur in both coding and non-coding regions of the pre-mRNA. Splicing in exon 9 and 10 produces unstable mRNA transcripts due to the AU-rich sequence in exon 10 and result in no functional protein production [149, 151-154]. Conversely, alternative splicing of the 5' end of exon 6 results in structural alterations of the translated CSF-1 protein causing the production of an intra-membrane glycoprotein [133]. Interestingly, the 68 kDa membrane-bound homodimer can be released following phorbol ester stimulation, which leads to the activation of protein kinase C, resulting in the proteolytic cleavage of a 44 kDa CSF-1 variant from the cell surface [155-157]. Transcripts of CSF-1 that possess the full length exon 6 lead to the translation of either a glycoprotein or proteoglycan CSF-1 variant, both of which circulate in the blood and are primarily produced by endothelial cells [133, 150, 158]. In cell culture both of these secreted forms of CSF-1 double in concentration approximately every 40 minutes, however the biologically relevant concentrations of CSF-1 range from 1 pM in bone marrow colony forming cells to 250 pM, at which the maximum CSF-1-induced proliferative effects are observed [139]. Commonly, the serum levels of CSF-1 observed in mice in humans ranges from 3 to 8 ng/mL [159-162].

It has been demonstrated that the N-terminal portion of CSF-1 is all that is required for CSF-1 mediated function, although post-translational modifications, such as those mentioned above, do impact the functional properties of the final molecule [132]. Interestingly, this N-terminal region of CSF-1 is the most conserved across species [50] and has been shown to possess important cysteine residues that are required for formation of the functional homodimer [163]. The presence of these cysteine residues allows for the formation of the biologically active homodimeric CSF-1 that ranges in size from 44 kDa to greater than 200 kDa [10, 132]. However, it has been demonstrated that post-translational modifications to CSF-1 are not required for function; this is highlighted by the usage of a prokaryotic recombinant CSF-1 molecule to perform early characterizations of CSF-1 function [164]. The alternative splice variants allow for an elegant system of CSF-1 regulation whereby functional effects can be induced by both direct contact of membrane bound CSF-1 with its receptor on the surface of the target cell or by circulating CSF-1 produced by cells elsewhere in the body [16, 165].

In addition to its roles in monopoiesis, CSF-1 also plays significant roles in bone metabolism, atherogenesis, lipoprotein clearance, and in female reproduction [4, 150, 166]. Moreover, CSF-1 is also a key regulator of macrophage function; it is a potent mediator of monocyte and macrophage activation increasing the chemotactic [167], phagocytic [168, 169], tumoricidal [170, 171] and antimicrobial activities such as the production of reactive oxygen and nitrogen intermediates [172-174]. CSF-1 also stimulates the production of several cytokines including G-CSF, GM-CSF, interleukin-1 (IL-1), IL-6, IL-8, TNFα, and interferons [150, 166]. Conversely, the absence of CSF-1 results in the osteopetrotic (*op/op*) mouse which lacks active CSF-1 production due to a null mutation in the coding region of the CSF-1 gene, leading to the generation of a biologically inactive truncated form of the cytokine [175, 176]. *Op/op* mice are deficient in osteoclasts and macrophages, have no teeth, and exhibit abnormal bone remodelling, osteopetrosis, low body weight, abnormal breast development, decreased fertility, and shortened life-span. These effects can be reversed by injection of neonatal animals with recombinant CSF-1 [177, 178].

The biological effects of CSF-1 are mediated by the high affinity CSF-1 receptor (CSF-1R) [179]. CSF-1R is a large integral membrane protein with an approximate M_r of 150 kDa. It is a member of the class III receptor tyrosine kinase family (RTK), possessing an N-terminal extracellular region composed of five immunoglobulin (Ig) domains, followed by a single transmembrane domain and two intracellular kinase domains which are involved in signalling [180, 181]. Other members of the RTK family include stem cell growth factor receptor (c-kit) [182], Flt3 [183, 184], platelet-derived growth factor receptor alpha (PDGFRα) [185, 186], and PDGFRβ [187] which all share a similar extracellular composition and intracellular signalling mechanism [133].

The CSF-1 receptor is expressed primarily on cells of the macrophage lineage [179] and has been used as a marker for cells that are or will eventually differentiate into monocytes and macrophages. Surprisingly, the CSF-1R has also been demonstrated to be expressed in cells of the granulocyte lineage [188]. However, these cells never produce functional receptor on their surface [150, 189] indicating that CSF-1R mRNA expression is not a reliable method for identifying monocytes or their progenitors. In addition to monocytes, macrophages and their progenitors, the CSF-1 receptor is also found on osteoclasts [190, 191], placental trophoblasts [192, 193], and mammary epithelial cells during lactogenic differentiation [194]. Furthermore, CSF-1R expression in astrocytes, can be induced following injury [195] or in myeloid leukemic blast cells [196], ovarian neoplastic cells [197, 198] and vascular smooth muscle cells [199, 200].

The human *fms* proto-oncogene (c-*fms*) encodes for the CSF-1 receptor. In mammals the gene is located on chromosome 5q33.3 [201, 202], is 58 kb in length, and composed of 22 exons and 21 introns [203]. Regulation of *c-fms* transcript expression occurs as a result of extracellular and intracellular stimuli primarily at the level of transcription. Two different tissue-specific promoters differentially dictate the transcription start site of the c-*fms* gene [194, 204, 205]. CSF-1R expression during pregnancy and lactation is under the control of the upstream promoter (which is located 350 bp from exon 1) and is regulated by the presence of sex steroid hormones [193, 206, 207]. CSF-1R expression on the surface of macrophages and their progenitors is under the regulation of the downstream promoter (located upstream of exon 2) [200, 205].

Further regulation of CSF-1R expression can be achieved by the regulation of lineage-specific transcription factors such as PU.1 [208-211], Ets1 [212], Ets2 [213], C/EBPβ [82], AML1 [82, 214], MITF [215], and c-myb [213, 216], all of which when expressed further monocyte development from progenitors at distinct junctures of development.

Interaction of the homodimeric CSF-1 molecules with the extracellular Ig domains of the CSF-1R results activation of downstream signalling via tyrosine phosphorylation. Binding of CSF-1 has been shown to be primarily mediated by the first two Ig domains of the receptor [188]. CSF-1 interaction with the CSF-1R results in dimerization of the receptors which leads to auto- and transphosphorylation of tyrosine residues (Y699, Y708, &723, Y809 and Y561 in humans [217], and Y697, Y706, Y721, Y807 and Y559 in the mouse [218-220]) in the intracellular portion of the receptor [221, 222]. After being phosphorylated, the CSF-1R can engage a number of intracellular signalling molecules possessing *src*-homology 2 (SH2) domains, which induce downstream signalling events. After the receptor has been activated and signalling has occurred, the CSF-1R molecules covalently dimerize [223], which leads to poly-ubiquitination of the cytoplasmic domain, kinase inactivation, phophotyrosine dephosphorylation, internalization via clathrin-coated pits and vesicles, targeting to lysosomes, and destruction of the receptor–ligand complexes [166, 221].

In the hematopoietic tissues, the CSF-1R molecule is only expressed on the surface of cells committed to the macrophage lineage. Stromal cells located in the bone marrow produce CSF-1 in specialized areas which results in niches of monopoiesis [179, 224]. As cells develop towards becoming a fully functional macrophage, their sensitivity to CSF-1 increases due to a steady increase in the expression of the CSF-1R on their surface [16]. Ultimately, this allows CSF-1 to stimulate the proliferation, differentiation, survival and activation of all stages of macrophage development as they become further committed to the macrophage lineage.

Regulation of CSF-1 function occurs at many levels. Besides having a short half-life [225], circulating CSF-1 can be effectively cleared by a process of CSF-1 receptor-mediated internalization followed by intracellular destruction of the growth factor [225-228]. It has been demonstrated that liver and splenic macrophages are capable of absorbing approximately 94% of the circulating CSF-1, leaving the remaining 6% to be filtered through the kidney [227]. That differentiated macrophages more efficiently degrade circulating CSF-1 suggests an elegant negative-feedback mechanism through which macrophage numbers can be effectively controlled. The primary mechanism of CSF-1/CSF-1R degradation is mediated by ubiquitin-protein ligase (c-Cbl). c-Cbl has been shown to increase the rate of ubiquitination and degradation of several receptor tyrosine kinases, including the receptors for epidermal growth factor, platelet-derived growth factor, and CSF-1 [229]. It is recruited to the plasma membrane upon the binding of CSF-1 to the CSF-1R after which c-Cbl associates with tyrosine 973 (mouse) or 969 (human) using its tyrosine kinase binding domain [229-231]. Association of c-Cbl with CSF-1R leads to ubiquitination of the ligand-receptor complex followed by internalization and degradation [232].

Further mechanisms that lead to inhibition of CSF-1 functions include the transphosphorylation of tyrosine residues in the receptor that are bound by molecules that contain *src*-homology 2 (SH2). This not only leads to downstream signalling events but also to recruitment of a number of negative regulators of CSF-1 action. P56^{dok-2}, a member of the Downstream of Tyrosine Kinases (dok) family of docking proteins, is rapidly phosphorylated in response to CSF-1 which results in negative regulation of macrophage proliferation [233]. Over expression of P56^{dok-2} inhibits proliferation of M-NFS-60 myeloid leukemia cells, and the inhibition of endogenous P56^{dok-2} expression in J774A.1 macrophage-like cells accelerates their proliferation [233]. These functions, although not completely understood, likely occur because P56^{dok-2} competes with other tyrosine kinase substrates, which prevents downstream signalling [234]. Another regulator of CSF-1 activity that associates with CSF-1R using its SH2 domain is the Suppressor of Cytokine Signalling 1 (SOCS1), which directly associates with Y697 and Y721 of the CSF-1R [235]. Following phosphorylation of tyrosine residues, SOCS1down-regulates proliferation-inducing signalling cascades by binding and subsequent inhibition of Janus kinase (JAK) family members [235, 236]. In addition, a number of phosphatases also negatively regulate CSF-1 receptor signalling. SHP-1 (Src-homology-2-domain-containing protein tyrosine phosphatase 1) regulates CSF-1 activity by direct interaction and dephosphorylation of the CSF-1 receptor. This was demonstrated by observation that macrophages from SHP-1 deficient motheaten (*me/me*) and motheaten viable (*me*^{v}/*me*^{v}) mice have an enhanced proliferative response induced by CSF-1 stimulation and have a hyper phosphorylated CSF-1 receptors [237].

From an evolutionary perspective, CSF-1R has been identified in a number of non-mammalian organisms including chickens [238, 239] and various teleost fish species [49, 240-242]. Furthermore, a teleost CSF-1 transcript was recently identified in the goldfish [245], zebrafish [50] and rainbow trout [243]. In most species of fish, the CSF-1R was used as a marker of myeloid cells, however a novel soluble version of the CSF-

1R has also been identified in the goldfish and has been shown to regulate cell proliferation and differentiation [49]. The soluble CSF-1 receptor was generated by alternative splicing of the mRNA species, which leads to the production of a truncated protein that has the extracellular ligand binding region, but lacks the cellular membrane anchoring domains. Recombinant goldfish CSF-1 was shown to induce the proliferation of sorted goldfish monocytes and induced their differentiation into functional macrophages. Addition of the soluble CSF-1R or an antibody to the CSF-1R abrogated both of these effects [50]. Akin to mammalian CSF-1, goldfish CSF-1 induced reactive oxygen intermediate production, enhance phagocytic responses and chemotaxis of goldfish PKM (see chapter 4). Teleost CSF-1 shares low amino acid identity with mammalian CSF-1; however both the goldfish and zebrafish CSF-1 predicted peptides have essential cysteine residues required for interaction with the CSF-1R. Cross-linking studies using the goldfish recombinant CSF-1 demonstrated that not only does goldfish CSF-1 form homodimers like its mammalian counterpart, but is also recognized by the soluble CSF-1R, confirming that the soluble receptor was of central importance to this novel teleost mechanism for regulation of CSF-1 function [49, 50].

2.4.2 Granulin

Pro-granulin (pgrn) is a high molecular weight growth factor [244] involved in tumorigenesis [245-247], regulation of mitosis [46, 248-251], wound repair [252], early embryonic development [253, 254], hypothalamic development [255-257], neurodegeneration [258-260], and certain non-neoplastic proliferative disorders. Granulins were first identified as small (6 kDa) peptides that were later demonstrated to be a product of the proteolysis of the larger pgrn molecule by leukocyte-derived elastase activity [261, 262]. Pgrn is known by several names, including granulin/epithelin precursor [263], acrogranin [264], PC cell-derived growth factor (PCDGF) [245] and progranulin [265]. All molecules that are considered granulins share a unique 12 cysteine motif that is arranged in 4 β-hairpins, stacked one upon another in a helical formation and connected by a central rod held together by disulphide bonds [244, 261]. Structurally, granulins are distinct from most growth factors, with exception of the epidermal growth factor/transforming growth factor-alpha family [266].

A functional receptor for pgrn or the small granulin peptides has yet to be identified, however it has been demonstrated that pgrn [267] and granulin peptides [268] can bind to membrane proteins. Chemical cross-linking studies identified a protein of approximately 120 kDa as a putative pgrn receptor on mink lung epithelial cells (CCL64), murine embryo fibroblasts, and the PC teratoma line [267]. On the other hand, cleaved granulin peptides cross-link a protein of approximately 140 kDa [268]. In both cases, Scatchard analysis indicated the presence of binding sites that had low affinity for both pgrn [267] and granulin peptides [268]. Yeast two hybrid analysis using Dlk as bait captured pgrn as the binding partner [269]. Dlk is a member of the epidermal growth factor-like homeotic membrane-protein family and it takes part in adipocyte and adrenal gland differentiation in part by acting as a negative regulator of Notch1 activation [270]. At approximately 40 kDa, Dlk is smaller than the pgrn-binding proteins identified by cross-linking. Interestingly, the membrane protein growth-arrest specific protein 1 (GAS-1) also binds to Dlk, and lower Dlk expression enhances pretumor-like formations in fibroblasts [269, 271]. Both of these actions are antagonistic to what has been observed for pgrn functions. These observations lead to hypotheses that Dlk may be a negative modulator of pgrn activity.

Despite the lack of knowledge of a putative granulin receptor, there has been detailed analysis of how pgrn and granulin peptides exert their biological effects. Pgrn has been shown to stimulate the phosphorylation of shc and p44/42 mitogen activated kinase (MAP kinase) in the extracellular regulated kinase signalling pathways (ERK), and phosphatidyl inositol-3 kinase (PI-3 kinase), protein kinase B/AKT, and the $p70^{S6kinase}$ in the PI-3 kinase cascade [252, 272, 273]. In addition, pgrn has been demonstrated to increase tyrosine phosphorylation of focal adhesion kinase (FAK) [274]. FAK mediates signalling to and from integrins and the actin cytoskeleton, and more importantly, it has been shown to be physically associated with the cytoplasmic domains of growth factor receptors, acting as their motility signal [275-278]. Thus, it has been proposed that FAK may facilitate communication between pgrn and integrin signalling [261]. Alternatively, there are reports of pgrn acting directly on the nucleus, bypassing the traditional signal transduction pathways. Pgrn binds to HIV Tat proteins [279], and to cyclin T_1[280] which has down-stream consequences on the phosphorylation of RNA polymerase II. Importantly, different granulin-repeat sections of the pgrn molecule (DE and E) have been shown to have differential binding affinities for Tat and cyclin T_1. In combination, granulin DE and E repress transcription from the HIV-1 LTR and gene expression from the viral genome, raising the possibility of developing granulin-based inhibitors of viral infection [281]. In certain cells, pgrn also affects the expression of mitotic cyclins. For example, in aggressive breast cancer cells, pgrn has been shown to increase the expression of cyclin D_1 which aids in driving the cell cycle into S-phase

[272]. In general, signalling of pgrn and the granulin peptides resemble the signalling pathways of well studied growth factors that employ tyrosine-kinase receptors; however, characterization of the exact mechanism of pgrn action must await the identification of the putative receptor(s) for this molecule.

Granulins are highly conserved among taxa. They have been identified in almost all metazoan phyla and plants that possess various proteases containing granulin cysteine motifs [282]. Depending on the species, the pro-granulin molecule may possess several signature cysteine motifs; however, in species where functions of granulins have been addressed, it is apparent that the pro-granulin molecule is not only functional on its own but also active as much smaller cleaved peptides. Moreover, it is becoming apparent that organisms such as fish (zebrafish, goldfish and tilapia) possess multiple granulin encoding genes [46, 253, 283], and that the functions of each of these granulin molecules are different [46, 253]. Interestingly, these additional granulin molecules found in fish exhibit differential expression patterns and some are expressed exclusively in hematopoietic tissues. Whereas mammalian progranulin genes are expressed almost ubiquitously, an expression pattern also seen in the fish progranulin genes [253], the smaller granulin transcripts, which possess only one and a half of the 12-cysteine repeats, are expressed to a much higher degree in the kidney and the spleen [46, 253, 265]. In the goldfish, a recombinant protein representing the putative protein encoded for by one of these smaller granulin transcripts induced proliferation of mononuclear cells enriched for cells of the monocyte/macrophage lineage. These cells appear to be the progenitors of functional monocytes and macrophages. Importantly, a polyclonal antibody specific for the recombinant goldfish granulin recognized native goldfish granulin of approximately the same size as the recombinant molecule in the cell-conditioned supernatants of monocyte/macrophage cultures [46]. These findings in fish mirror studies performed in mice which have shown granulin peptides and pgrn to be associated with myeloid cells [265, 284]. In fact, the mammalian pgrn transcript was highly expressed in monocyte-derived cells [285-287]. Furthermore, it was found in myeloid leukemic cells that were undifferentiated progenitors of granulocytes and monocytes [288, 289], as well as in mature myeloid cells [252, 285] and CD34$^+$ bone marrow cells [288]. These observations suggest that mammalian pgrn, and possibly its cleavage products, may also be involved in monopoiesis as has been first demonstrated for teleosts.

The degree of evolutionary conservation between molecules classified as granulins is due in large part to the 12-cysteine motif used to classify molecules in this group. Commonly, this motif is all that is shared between granulin-like molecules of very divergent species. For example, granulins identified in invertebrate organisms such as the mollusc *Patinopecten yessoensis* [290], the migratory locust *Locusta migratoria* [291], the sand worm *Nereis diversicolor* [292], the laval sphinx moth *Manduca sexta* and the mosquito *Aedes albopictus* [293], all share very low amino acid identity with mammalian or fish granulins (between 28-34%), with the primary regions of sequence identity occurring in the 12-cysteine motif. Although the intervening sequences are very different, granulin molecules of many different organisms share common functional properties such as being anti-bacterial in the horse [294] or the sand worm [292], or inducing proliferation in both fish [46] and mammals [295, 296] . This overlap in function with little sequence similarity except in specific regions of the molecule leads to questions about the functional requirements for a granulin molecule. The unique shape conferred to granulin molecules by the 12-cysteine motif [244] is often referred to as the functional requirement for granulin-like molecules, since all functional studies of granulins have been done with molecules possessing some arrangement of this motif. That the 12-cysteine motif induced similar functions across species boundaries was illustrated by a study where a recombinant goldfish granulin molecule was mitogenic for insect cells of *M. sexta* and *A. albopictus* [293]. Cleavage motifs of the mammalian pgrn protein have been demonstrated to have pleiotropic functions, with certain granulin peptides inducing proliferation [297], and others inducing pro-inflammatory responses [262]. More research is needed to assess the different roles of pgrn cleavage products, due to the contradictory nature of these preliminary observations.

Granulins are relative "new-comers" when compared to other well studied growth factors such as fibroblast growth factor and insulin-like growth factor-1. The impressive array of functions that have been attributed to granulins, ranging from cancer onset and progression, neural degeneration, embryonic development and hematopoiesis, suggests that granulins are important growth factors for maintenance of homeostasis and host response to sudden insult. The extreme degree of structural conservation of granulins among metazoan suggests that this molecule is a major player in the physiology of organisms.

2.4.3 Leukemia inhibitory factor (LIF)

Leukemia inhibitory factor (LIF) belongs to the IL-6 cytokine family that also includes interleukin 6 (IL-6) and 11 (IL-11), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1) and oncostatin M (OSM). LIF has a diverse array of effects, including the induction of proliferation in some hematopoietic cells, bone formation, hormone production, the production of acute phase proteins, formation and survival of neurons, and embryonic development [298]. Perhaps the most well studied aspect of LIF biology is its involvement in the survival, formation and repair of neurons and the maintenance of neural and embryonic stem cells [299-301]. LIF promotes the self-renewal of mouse embryonic stem cells [302], mouse embryonic neural stem cells [299, 302-305], and human embryonic neural stem cells [306]. Although the precise mechanisms behind the retention of stem cell multipotency are not known, LIF receptor (LIFR)/gp130 signalling has been shown to regulate the expression of Notch1 [307], which was shown to be important for the maintenance of neural stem cells [308]. Moreover, LIF expression is commonly associated with neural injury, and has been shown to be an essential stimulus for proliferation of neuronal progenitor cells after injury [309]. In addition, LIFR/gp130 signalling was shown to be instrumental in the differentiation of neural progenitors into astrocytes [310].

LIF was originally identified in medium conditioned by Krebs-II ascites tumour cells [311] and was subsequently cloned from a murine T-lymphocyte cDNA library. Originally, LIF was characterized as a factor that was able to induce macrophage maturation and arrest the self-renewal of undifferentiated murine myeloid leukemia, M1 cells [312]. This early inhibitory role of LIF on the proliferation of leukemia cells earned it its name, and early studies using LIF failed to demonstrate its ability to induce hematopoietic colony formation *in vitro* [313]. Later studies however, demonstrated that LIF used in combination with IL-3 or Flk-2 ligand was able to enhance hematopoietic colony formation *in vitro* resulting in mouse megakaryocytes [314] and monocytes/macrophage progenitor cells, with a tendency to form monocyte-derived dendritic cells [315]. Although the results of these studies suggested LIF may have a role in enhancing the proliferation of hematopoietic stem cells [48, 316-318], to date, LIF has not been used for the routine *in vitro* cultivation of mammalian hematopoietic stem cells.

In addition to functioning as a hematopoietic modulator of growth and development, LIF is also an important neuropoietic cytokine. LIF has been shown to be an essential player in response to neural injury. In the case of neural damage, LIF transcript expression is rapidly upregulated [319-322]. During this localized event, LIF has been shown to regulate the phenotype of neurons [323-325] and to be involved in the co-ordination of the inflammatory response orchestrated by the astrocytes, oligodendrocytes and microglia at the injury site [321, 325, 326]. After resolution of the injury, LIF was shown to induce the proliferation of neuronal progenitors [299, 309] in part by stimulation of Notch1 expression [304, 307]. In addition, LIF has been shown to be involved in the cholinergic differentiation of sympathetic neurons *in vitro* [327] and *in vivo* [328]. In contrast, studies in mice deficient in LIF or another important neuromodulatory IL-6 family cytokine, CNTF, suggested that neither LIF nor CNTF were essential for cholinergic differentiation of sympathetic neurons [329]. An important discovery clearing up this apparent discrepancy was that in fact LIFR is required for the cholinergic differentiation activity observed after stimulation with LIF or CNTF and that LIF or CNTF stimulated this receptor-activated process in conjunction with other cytokines of the IL-6 family [330]. LIF has also been reported to play an important role in embryogenesis, however LIF-/- mice have very few abnormalities aside from the observation that LIF-/- female mice cannot become pregnant [316, 331], a deficiency that was overcome by injection of recombinant LIF [332].

The shared receptor complexes used by the IL-6 cytokine family confer a high degree of redundancy to this entire family of biomolecules. LIF, CNTF, CT-1 and OSM are recognized by heterodimeric receptor complex that includes leukemia inhibitory factor receptor (LIFR) and gp130, whereas IL-6 and IL-11 bind a homodimeric receptor composed of two gp130 molecules [333-335]. In the case of LIF, LIF-LIFR interactions occur first and are low affinity interactions with a K_d of 1-4 x 10^{-9}. Association of this LIF-LIFR complex to the cell surface gp130 molecule converts the low affinity interactions between LIF and LIFR into high affinity interactions with a K_d of approximately 10-200 x 10^{-12} [336, 337]. Dimerization of the receptor subunits induces downstream signalling cascades that function largely via the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) and the MAP (mitogen-activated protein) kinase pathways [338-342].

Jak 1 and Jak 2 associate with the cytoplasmic domains of both LIFR and gp130 in the absence of bound ligand, within the 74 membrane-proximal amino acids of each receptor, and upon ligand binding, the Jaks are autophosphorylated and activated [334]. Disruption of Jak 1 has been shown to abrogate signalling from the gp130 receptor component and thus stop any LIF-mediated signalling [343]; similarily, over expression of a Jak 1 dominant-negative mutant *in vitro* also abrogated LIF responsiveness. Targeted disruption of Jak 2 or over expression of a Jak 2 dominant negative mutant significantly affected LIFR signalling [344-346], suggesting that Jak 1 and 2 are significantly involved in LIFR signalling. In addition to Jak pathway signalling, specific phosphorylated tyrosine residues in the cytoplasmic domain of LIFR and gp130 are able to act as docking sites for the SH2-domains of STAT proteins [347-350]. These interactions allow for receptor homo/heterodimerization and subsequent phosphorylation of STAT1, STAT3 or STAT5a (reviewed in [351]). The response initiated by Jak/STAT activation by LIF was shown to be cell specific [352], and that specific tyrosine residues are required for proper STAT function. The YXXQ motif provides a binding region for the SH2-domain of STAT [348, 350], and STAT binding to the cytoplasmic tail of the receptor subunits results in a closer association between the STATs and the Jak kinases which results in phosphorylation of tyrosine residues of the STAT molecules [353, 354]. Over-expression of STAT3 dominant negative mutants [355, 356] abrogated LIF-induced differentiation of M1 cells [357] and reduction of activated STAT3 levels inhibited LIF-induced maintenance of pluripotent embryonic stem cells [358], suggesting that STAT3 plays an important role in LIF-mediated signalling in a variety of cell types.

In addition to signalling via the Jak/STAT cascade, LIF has also been shown to initiate the Ras-MAPK pathway. LIF has been shown to stimulate Shc [347], Ras [347, 359], Raf-1 [360], MAPKK [361], Erk1, and Erk2 activity [355, 360-362]. It has been demonstrated that SHP-2 was required for LIFR/gp130-mediated activation of MAPK and that the ability of LIF to initiate MAPK tyrosine phosphorylation was dependent on the cell type [360-364]. Activation of SHP-2 by LIF required the presence of specific tyrosine residues located on both gp130 (Y118) and LIFR (Y115) [365-368], and deletion of either of these tyrosine residues resulted in an absence of LIF-induced MAPK activity [347, 362]. In addition to SHP-2 being essential for the activation of LIFR/gp130 by LIF, phosphatidylinositol (PI) 3-kinase was also shown to be an essential component of LIF-induced MAPK activation, since PI-3-kinase inhibitor wortmannin inhibited LIF- and IL-6-induced activation of MAPK [369-371].

Given the many functions regulated by LIF, it is intuitive that LIF action must be tightly regulated. Regulation of LIF occurs on multiple levels, including regulation of LIF expression, sequestration and removal of free LIF, and control of LIFR/gp130 signalling events. LIF transcript constitutive expression levels are generally low in all tissues and expression is usually undetectable by Northern blot without first inducing LIF expression using pro-inflammatory molecules. Bacterial lipopolysaccharide (LPS) [372] induces LIF expression as do the inflammatory cytokines IL-1 [373-375], TNF-α [373-375], and IL-17 [376]. Conversely, anti-inflammatory cytokines such as IL-4 [377] and IL-13 [376] or glucocorticoids [378, 379] inhibit the expression of LIF. In addition to regulation of LIF transcript expression, mechanisms are in place to control the amount of translated LIF. Although the LIFR is commonly expressed as a membrane bound protein, it can also be expressed as a soluble receptor which lacks the transmembrane and cytoplasmic domains. In the mouse, the membrane bound receptor is derived by alternative splicing that occurs by skipping an exon that contains a translation termination codon and is specific for the soluble form [380, 381]. Expression of the soluble receptor is often highest during pregnancy [382, 383] and gestation [381], and is commonly seen to be confined to the liver where an alternative promoter in the 5' exon 1 is used [384]. Importantly, both murine and human soluble LIFR inhibit LIF actions *in vitro* and *in vivo* [385-387], suggesting that the soluble receptor may act as an important regulator of LIF action during inflammation or development. Interestingly, free LIF can also be regulated by the mannose-6-phosphate/insulin-like growth factor II receptor (Man-6-P/IGFII-R).

This receptor has been shown to have moderate affinity for the glycosylated but not deglycosylated LIF [388]. Binding of glycosylated LIF to the membrane bound, as well as a soluble version of the Man-6-P/IGFII-R, results in no down-stream functional effects on any of the cells it has been studied. LIF binding to the Man-6-P/IGFII-R results in rapid internalization of the receptor-ligand complex and degradation of LIF, thus reducing LIF bioavailability [388-390].

The regulation of LIF activity not only occurs at the level of LIF bioavailability but also at the level of receptor signalling. Negative feedback regulators of LIFR/gp130 activation of the Jak/STAT signalling pathway act to prevent the final stages of LIF activity. The SH-2-containing protein tyrosine phosphatase-2 (SHP-2), mentioned previously as a molecule involved in activation of the MAPK pathway, also acts as a blocker of LIF-induced effects. LIF stimulates tyrosine phosphorylation of SHP-2 which subsequently interacts with tyrosine 118 (Y118) in the gp130 receptor subunit [391-394]. Mutations in gp130 affecting this tyrosine residue or over expression of dominant negative SHP-2 variants have been shown to enhance LIF-induced effects [368, 395, 396]. In addition to SHP-2, suppressors of cytokine signalling (SOCS) and another family of negative regulators, called protein inhibitors of activated STAT (PIAS), can also inhibit signalling by LIFR/gp130. SOCS-1 and/or SOCS-3 have been shown to inhibit the signalling of numerous Jak/STAT signalling cytokines including the IL-6 family cytokines [397]. For example, over expression of SOCS-3 prevents LIF-induced phosphorylation of gp130 and STAT-3, thereby inhibiting downstream STAT-3 mediated events [351, 398]. SOCS-1 and SOCS-3 have been shown to inhibit Jak/STAT mediated signalling by either directly (SOCS-1) [397, 399] or indirectly (SOCS-3) [400] inhibiting

Jak2 activation. In the case of SOCS-1 this is achieved by directly binding to Jak2 [401]. PIAS1 and PIAS3 also prevent signalling via the Jak/STAT pathway, however PIAS1 and PIAS3 act by interacting directly with activated STAT-1 and STAT-3, respectively, thereby inhibiting their downstream actions [402].

From an evolutionary perspective, the most ancestral members of the IL-6 cytokine family have been characterized in teleost fish; and genetic evidence indicates that they may be present in jawless fish. IL-6 family cytokines have been identified in the carp [403, 404], goldfish [48], zebrafish [404, 405], Japanese flounder [406], tiger puffer [407], green spotted puffer, stickleback [408], and rainbow trout [409, 410], and LIFR and gp130 molecules have been identified in goldfish, and zebrafish [47]. The characterization of teleost IL-6 family members has been focused on gene transcript expression analysis, mostly of cytokines that appear to be orthologs of either IL-6 or IL-11. More difficult to classify are the IL-6 family members that appear to be orthologs of the LIFR-specific cytokines.

Most of the work on the orthologs of the LIFR-specific cytokines was done on a molecule named M17 in teleosts, which has been shown to have similar expression patterns to mammalian LIF and CNTF. M17 had closest amino acid identity to CNTF, suggesting that teleosts possess at least two LIFR-specific orthologs. Identification and functional analysis of a goldfish M17 (called gLIF here after) transcript showed that it was highly expressed in the brain and kidney, and was able to induce the differentiation of goldfish monocytes into macrophages and enhance the proliferation of goldfish monocyte/macrophage progenitor cells in combination with cell-conditioned supernatants. Furthermore, analysis of zebrafish M17 suggested that it was syntenic to mouse and human LIF and OSM [48].

The examination of the expression and function of zebrafish *m17* (called *lif* here after) and zebrafish *lifr* (called *lifr* here after) during zebrafish embryogenesis and in the adult fish showed that *lif* is expressed as early as 12 hours post-fertilization (hpf) and continues to be expressed into adulthood where it is highly expressed in the brain and kidney. Zebrafish *lifr* is expressed as early as 24 hpf and is highly expressed in the gill, brain, kidney and spleen. *In situ* hybridization analysis indicated that both *lif* and *lifr* are expressed in the developing forebrain and notochord. Morpholino-mediated knockdown of *lif* and *lifr* resulted in obvious phenotypic defects in the *lifr* morpholino-injected embryos by 48 hpf. Zebrafish *lifr* morphants exhibit significant defects in the trigeminal, facial and vagal branchiomotor neurons, visualized through the use of the Isl1-GFP WIK transgenic zebrafish line. Zebrafish *lifr* morphant embryos also exhibit improper axonal development, shown through labelling with an anti-acetylated tubulin antibody, and display severe hydrocephaly by 48 hpf. Zebrafish *lif* morpholino-injected embryos display no detectable phenotypic differences from uninjected controls. This is the first evidence that a LIFR-like molecule plays a role in the neural development of teleosts [405]. The identification of another putative LIFR-specific cytokine termed M17 homolog (MSH) resembles both goldfish LIF and zebrafish *lif* except that it possesses two more cysteine residues, likely changing the function of the molecule encoded by this gene, which remains to be determined [408]. In addition, goldfish LIFR has also been identified, suggesting that the LIF/LIFR/gp130 signalling mechanism may be conserved in teleosts [47].

2.5 Summary

The majority of studies that characterized growth factors and their effects used mouse model systems. Very little work has been done to characterize the growth factor repertoire in early vertebrates such as fish, and even less work has been done to compare the functions of early vertebrate growth factors to those of mammals. Recently, a number of growth factors have been identified in fish, allowing comparative assessment of their function between early and later vertebrates. The main objective of this thesis research was to further the understanding of the molecular control of myelopoiesis in fish. I focused my work on three growth factors and their receptors that have been shown to influence myelopoiesis in mammals. They were macrophage colony stimulating factor, granulin, and leukemia inhibitory factor.

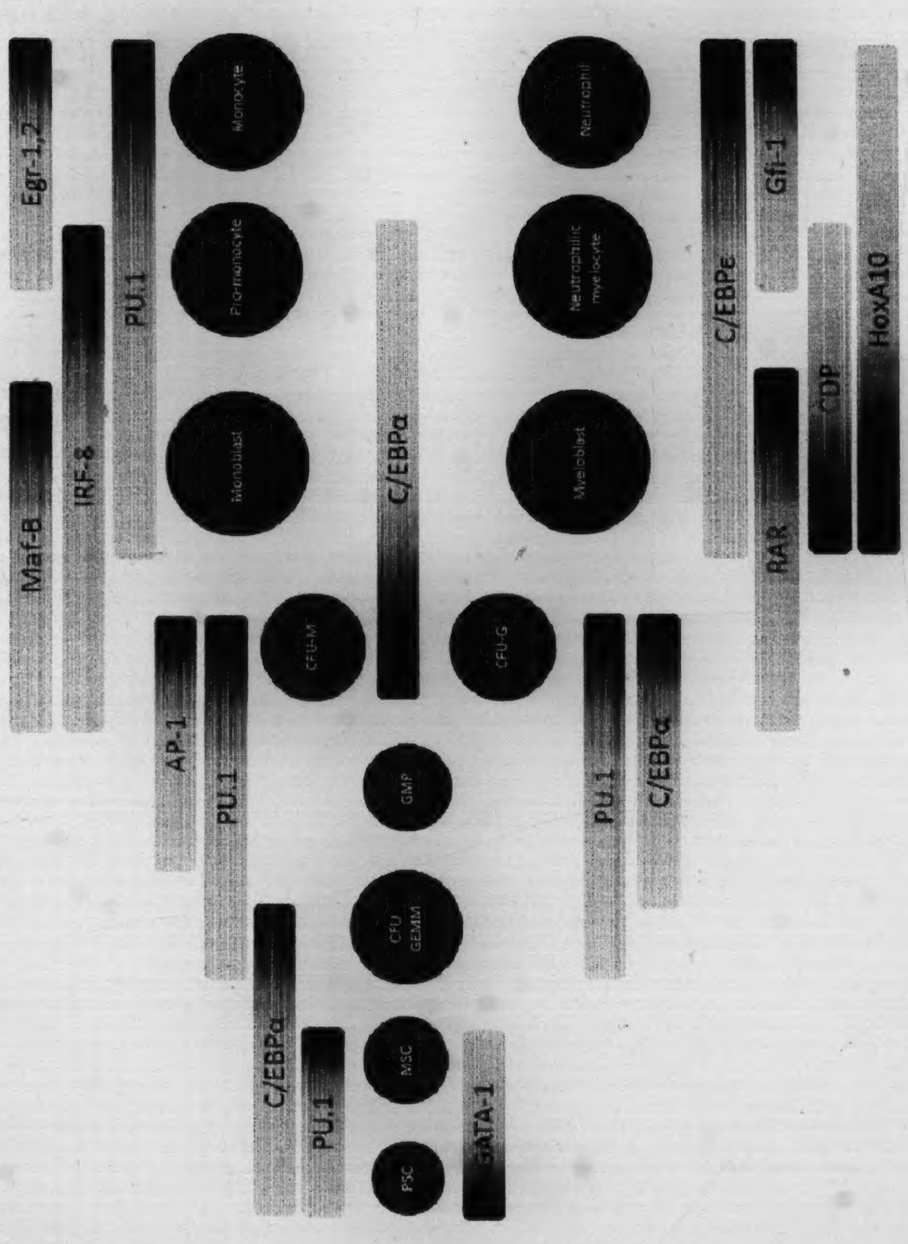
Egr-1,2
PU.1
Monocyte
Pro-monocyte
IRF-8
Maf-B
Monoblast
C/EBPα
Neutrophil
Neutrophilic myelocyte
Myeloblast
C/EBPε
Gfi-1
RAR
CDP
HoxA10
CFU-M
CFU-G
AP-1
PU.1
PU.1
C/EBPα
GMP
CFU GEMM
C/EBPα
PU.1
MSC
PSC
GATA-1

Figure 2.1 Summary of the transcriptional regulation of myelopoiesis. Transcription factors indicated in red are involved in driving differentiation events whereas those shown in blue are inhibitory. Colour intensity indicates increasing or decreasing amounts of each factor during development. Intensity of shading indicates higher expression levels.

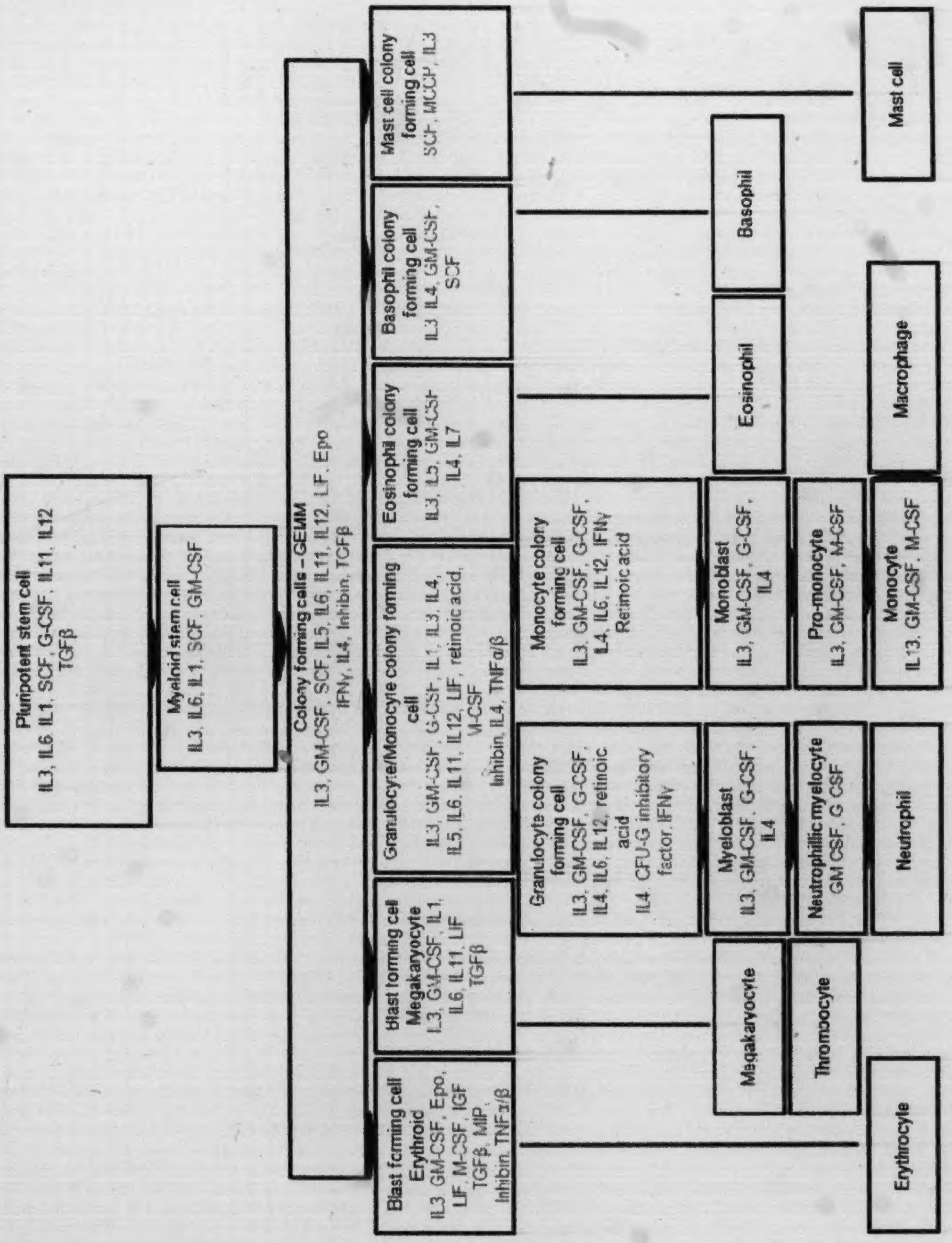
Pluripotent stem cell
IL3, IL6, IL1, SCF, G-CSF, IL11, IL12
TGFβ
Myeloid stem cell
IL3, IL6, IL1, SCF, GM-CSF
Colony forming cells - GEMM
IL3, GM-CSF, SCF, IL5, IL6, IL11, IL12, LIF, Epo
IFNγ, IL4, Inhibin, TGFβ
Blast forming cell
Erythroid
IL3, GM-CSF, Epo,
LIF, M-CSF, IGF
TGFβ, MIP,
Inhibin, TNFα/β
Blast forming cell
Megakaryocyte
IL3, GM-CSF, IL1,
IL6, IL11, LIF
TGFβ
Granulocyte/Monocyte colony forming cell
IL3, GM-CSF, G-CSF, IL1, IL3, IL4,
IL5, IL6, IL11, IL12, LIF, retinoic acid,
M-CSF
Inhibin, IL4, TNFα/β
Eosinophil colony forming cell
IL3, IL5, GM-CSF
IL4, IL7
Basophil colony forming cell
IL3, IL4, GM-CSF,
SCF
Mast cell colony forming cell
SCF, MCGF, IL3
Granulocyte colony forming cell
IL3, GM-CSF, G-CSF,
IL4, IL6, IL12, retinoic acid
IL4, CFU-G inhibitory factor, IFNγ
Monocyte colony forming cell
IL3, GM-CSF, G-CSF,
IL4, IL6, IL12, IFNγ
Retinoic acid
Myeloblast
IL3, GM-CSF, G-CSF,
IL4
Monoblast
IL3, GM-CSF, G-CSF,
IL4
Neutrophilic myelocyte
GM CSF, G CSF
Pro-monocyte
IL3, GM-CSF, M-CSF
Neutrophil
Monocyte
IL13, GM-CSF, M-CSF
Megakaryocyte
Thrombocyte
Erythrocyte
Eosinophil
Basophil
Macrophage
Mast cell

Figure 2.2 Flow diagram of cytokines and factors important in hematopoiesis and myelopoiesis. Cytokines indicated in red drive the current cell into the follow lineage commitment stage. Cytokines indicated in blue inhibit the process of cell differentiation. Only myelopoietic events are shown in detail after the CFU-GEMM stage.
]

Chapter 3

Materials and Methods

3.1 Animals

3.1.1 Goldfish

Goldfish (*Carassius auratus*) were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20°C in a flow-through water system on a simulated natural photoperiod, and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments. All of the fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes were used. All protocols were carried out in compliance with the guidelines stipulated by the Canadian Council for Animal Care (CCAC) and the University of Alberta.

3.1.2 Zebrafish

Wild-type AB and Isl1-GFP WIK transgenic zebrafish (*Danio rerio*) embryos were raised at 28.5°C, and collected and staged as previously described [411, 412]. Embryos and larvae were anaesthetized in 0.02% tricaine (MS-222; Sigma Chemical, St. Louis, MO) in phosphate buffered saline (PBS) prior to all procedures. To block pigment formation in embryos and larvae, 0.003% phenylthiourea (PTU) was added to the embryo medium [412] at 20 hours post fertilization (hpf).

Embryos were fixed in 4% paraformaldehyde-phosphate buffered saline at 4°C overnight and tissues were washed several times in PBST (PBS and 0.1 % Tween-20) prior to *in situ* hybridization.

3.2 Cells

3.2.1 Goldfish macrophages

Isolation of goldfish kidney leukocytes and the generation of primary kidney macrophages (PKM) were performed as previously described [24, 43]. Briefly, goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (18-20 $\times 10^6$ cells from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium and 5 mL of cell-conditioned medium (CCM) from previous cultures. The culture medium (MGFL-15) used for cultivation of PKM, has been described previously [413]. Complete medium contained final concentrations of 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 10% newborn calf serum (Hyclone), and 5% goldfish or carp serum.

The kinetics of PKM growth in culture were similar to those reported for mammalian macrophages derived from bone marrow cultures in the presence of conditioned medium from the L-929 fibroblast cell line [414]. PKM cultures undergo a lag phase of growth during the first 4 days of cultivation. Both adherent and non-adherent cells were present in these cultures. Adherent cells spread extensively on tissue culture flasks and often formed multi-nucleated giant cells. The non-adherent cells grew in clusters and attained significant cell densities in culture (greater than 5×10^5 to 1×10^6 cells/mL). PKM cultures were composed of a heterogeneous population of cells, as determined by flow cytometry, morphology, cytochemistry, and function. Three distinct macrophage subpopulations are a feature of PKM cultures which represent macrophages subsets temporally arrested at distinct differentiation junctures in development: the early progenitors, the monocytes and mature macrophages [24, 43, 44, 415, 416]. PKM cultures were incubated at 20°C until the cells were at a stage of active growth (proliferative phase) or a non-proliferative stage (senescence phase) typically 6 and 10 days post cultivation, respectively.

3.2.2 Goldfish fibroblast CCL-71 cell line

Goldfish CCL-71 fibroblasts (ATCC) were grown in DMEM supplemented with 15% FCS and 5% carp serum at 20°C with no additional CO_2. Cells were grown to confluence at which time the supernatant was removed and 3 mL of 0.25% trypsin-EDTA was added to the cells. After 5 minutes of gentle agitation, 7 mL of medium was added to the cultures and 4 mL of the cell suspension was added to 16 mL of medium and placed in a new flask. The supernatants from CCL-71 were stored at 4°C, until used in the experiments.

3.2.3 Insect cells

Sf9, KC and Hi-5 insect cells (Invitrogen) were used for production of recombinant proteins. Cells were grown in ESF-921 medium at 27°C (Culture Systems, Inc) until they reached confluence, at which point they were dislodged from the flask bottom by agitation of the flask, the cells were harvested and re-suspended in 18 mL of ESF-921 medium. Stocks for cultivation were generated by freezing 1 x 10^6 cells in 1 mL of medium containing 20% DMSO in liquid nitrogen.

3.2.4 *Mammalian cells*

Chinese hamster ovary (CHO) cells (ATCC) were grown in DMEM supplemented with 10% FBS. Cells were grown at 37°C in 5% CO_2 atmosphere until they reached confluence, at which point the medium was removed and the cells were incubated with 3 mL 0.25% Trypsin-EDTA solution to detach adherent cells. Seven millilitres of medium was then added to the cell suspension, which was diluted further (1/10) by adding 18 mL of new DMEM medium prior to cultivation.

3.3 Cell bioassays

3.3.1 *Isolation of goldfish primary kidney leukocytes and generation of mitogen-stimulated kidney leukocyte conditioned supernatants*

Isolation of goldfish kidney leukocytes and the generation of PKM were performed as previously described [416]. Macrophage-activating factor (MAF) supernatants were prepared using protocols described previously [416, 417]. Briefly, goldfish kidney leukocytes (4 x 10^6/mL, 20 mL cultures) were incubated overnight (~18 hours) in medium containing 5% goldfish serum. Following phorbol myristate acetate (10 ng/mL), concanavilin A (10 µg/mL), and calcium ionophore A23187 (100 ng/mL) stimulation, leukocytes were washed 3 times with 25 mL Hanks balanced salt solution. The remaining cells were sub-cultured in fresh medium supplemented with 5% goldfish serum and incubated for 72 hours at 20°C prior to use. These supernatants contained a complex mixture of factors that have been functionally characterized and shown to induce antimicrobial responses of goldfish macrophages [416, 417].

3.3.2 *Detection of nitric oxide intermediates in goldfish macrophages*

Induction of a nitric oxide (NO) response in goldfish macrophages was determined by seeding goldfish monocytes/macrophages at a concentration of 5 x 10^4 cells/well in 50 µL in 96 well plates (Costar). Macrophages were then treated and allowed to incubate for 72 hours and nitrite concentration was determined using the Griess reaction [12]. Seventy five microlitres from each well was transferred to a new 96 well plate and to each well, 100 µL of sulfanilamide (Sigma) (dissolved in 2.5% H_3PO_4) followed immediately by 100 µL of 0.1% *N*-naphthyl-ethylenediamine (Sigma) (dissolved in 2.5% H_3PO_4) was added. The plate was incubated for 2 minutes and then read using a microplate reader at an absorbance of 540 nm. The approximate concentration of nitrite was determined from a standard curve generated using known concentrations of sodium nitrite.

3.3.3 *Flow cytometric analysis of primary goldfish macrophage cultures*

Goldfish macrophages were sorted into progenitor cells, monocyte and macrophage subpopulations on day four post-isolation. They were seeded into 12 well plates at a concentration of 3 x 10^6 cells/well in 3 mL of incomplete medium and subjected to different treatments. Treatments were re-applied on alternating days to data collection. Every 24 hours after treatment, the cells cultures in each well were analysed using a FACS Calibur flow cytometer (Becton Dickinson). Analysis was performed by measuring the forward (size) and side (internal complexity) scatter light patterns of the cells for each treatment group.

3.3.4 Chemotaxis assay

Test chemo-attractants in 200 μL were applied to the lower wells of a leucite chemotaxis chamber (Nucleoprobe Corp.) and overlaid with polycarbonate membranes (5 μm pore size; Nucleopore Corp.). To the upper wells, 5 x 10^5 of four to six-day-old PKM cells were added and the chamber was incubated for 4 hours at 20°C. Following this incubation, the contents of the top wells were aspirated and the filters were removed and mounted bottom-side-up on microscope slides. These were air dried and fix-stained using Wright's solution. Chemotactic activity was determined as the total number of cells counted in twenty randomly selected fields of view using the 100X objective. In order to measure chemokinesis, the highest concentrations of chemo-attractant were applied to both the upper and lower wells of the chemotaxis apparatus. The optimal chemotactic concentration for the control treatments was pre-determined in a preliminary chemotaxis experiment [418].

3.3.5 Phagocytosis assay

Four to 6 day PKMs were seeded into individual wells of 96 well plates at a density of 3 x 10^5 cells/well. PKM were exposed to different treatments and a 10:1 ratio of fluorescent beads (2.0 μm diameter YG, Polysciences) to cells was added to each well in a final volume of 100 μL/well. The assays were incubated for 18 hours and subsequently treated with trypsin-EDTA (0.05% Trypsin, Gibco) (40 μL /well) for 5 minutes at room temperature in order to remove cells from the bottom of the wells and non-ingested beads from cell surfaces. Cells were then suspended in 850 μL of incomplete medium to inactivate the Trypsin and spun down (100 x *g*, 15 minutes at 4°C) over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose in order to separate the cells from non-ingested beads. FACSs analysis of the cultures was performed under pre-optimized instrument settings using a FACSCalibur flow cytometer.

3.3.6 Nitro blue tetrazolium reactive oxygen production assay

Four to 6 day PKMs were seeded into 96 well plates at a density of 3 x 10^5 cells/well and exposed to different treatments for 5 or 18 hours. Following incubation, NBT (2 mg/mL, Sigma) / PMA (final conc.100 ng/mL) in PBS was added into the wells and the plates incubated at room temperature for 30 minutes. The plates were centrifuged at 300 x *g* for 5 minutes, the supernatants removed, and cells fixed with absolute methanol. Unreduced NBT was removed by washing with methanol and reduced NBT was dissolved with 2M KOH. DMSO was added to induce the colorimetric response and the plates were read at 630nm using a microplate reader. Readings from cells alone (no PMA) were subtracted from experimental group values to factor in background NBT reduction.

3.3.7 Measurement of cellular proliferation

The effect of goldfish growth factor addition on macrophage proliferation was assessed using the Cell Proliferation ELISA BrdU colorimetric assay (Roche). Macrophages were grown as described above and sorted using a FACS Calibur flow cytometer based on size and complexity. Progenitor cells, monocytes and macrophages were then counted and seeded at a density of 1x 10^4 cells/well in 96-well culture plates (Falcon). Cells were seeded in 50 μL of incomplete culture medium and treated with

BrdU labelling reagent at a concentration of 15 μM in combination with a known concentration of recombinant growth factor diluted in incomplete medium. Cell proliferation was measured every 2 days from the day 0 time point. The reaction was developed according to the manufacturer's specifications and optical densities determined at 450 nm using a microplate reader. The colorimetric reaction was found to be directly proportional to the number of proliferating macrophages in culture. Recordings from the untreated cells were subtracted from the experimental groups to account for the ability of PKM cells to proliferate in the absence of exogenous growth factor [44].

3.3.8 In vivo BrdU analysis of cell proliferation and growth factor effect

Fish were housed in holding tanks for 1 week prior to intraperitoneal injection with BrdU labelling reagent (Roche) at a concentration of 1mL of labelling reagent per 100 g of body mass, as per the manufacturer's protocols. A fish injected with 1X PBS served as a sham-injected control and was compared to a fish injected with 25 ng of recombinant goldfish CSF-1. The concentration of CSF-1 was selected to most accurately mimic the concentration used in previous *in vitro* assays of proliferation and differentiation, and was based on exsanguination volumes achieved from similar sized fish of approximately 2.5 mL of total blood. Fish were maintained in holding tanks for 2 days after treatments before being exsanguinated and having their kidney removed. Primary kidney leukocytes were isolated as mentioned in this chapter and peripheral blood leukocytes were separated from red blood cells to assess the ratios of different immune cells in the blood, and developmental profile of primary kidney cells. To purify peripheral blood leukocytes two hundred microlitres of blood was taken and mixed with 800 µL of heparinised 1X PBS to prevent clotting. Cells were then separated using a 51% percoll gradient centrifugation at 300 x *g* for 5 minutes and the supernatants and cell interface layer were removed. The cells were washed in 5 mL of 1X PBS for 5 minutes at 300 x *g* and the pellet was then re-suspended in 4.5 mL deionized milli-Q water, to remove any remaining red blood cells, and incubated for one minute before addition of 500 µL of 10X PBS. The cells were then centrifuged at 300 x *g* for 5 minutes and re-suspended in denaturant as per the manufacturer's specifications. The final cell population was suspended in 500 µL of 1X PBS and analyzed by flow cytometry using size and internal complexity paraeters, as well as fluorescence due to the incorporation of the BrdU labelling reagent. Populations were compared to pre-injection controls and sham-injected controls. Primary kidney cultures were generated as described in this chapter.

3.3.9 Growth of primary goldfish macrophages induced by CCL-71 cell line supernatants

Supernatants from CCL-71 cultures were tested for their ability to induce proliferation in goldfish progenitor cells, monocytes and macrophages. Cells were cultured as mentioned above and then, on day 1 post-isolation, the cells were counted using a haemocytometer and diluted to a concentration of 1 x 10^6 cells/mL. The cells were seeded in 96 well plates at 1 x 10^4 cells/well in 50 µL. The cells were incubated for 1 hour and the following experimental groups were set up: incomplete medium, CCM, CCL-71 supernatants, 10 ng/mL CSF-1, CCL-71 supernatants with 50 ng/mL sCSF-1R, or 10 ng/mL CSF-1 with 50 ng/mL sCSF-1R. The cells were then incubated with the BrdU labelling reagent and assayed for proliferation as described in BrdU assay section of this chapter.

3.4 Molecular assays and *in silico* analysis

3.4.1 Primers

All primers used for experiments outlined in this thesis are listed in table 3.1 at the end of this chapter.

3.4.2 Construction of cDNA libraries of primary kidney macrophages

Complementary DNA libraries were constructed from 2 μg of proliferative or senescence phase PKM poly (A)+ RNA by directional ligation of PKM cDNA into lambda ZAP bacteriophage using a ZAP cDNA synthesis kit, and the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Non-amplified libraries were prepared as they provided a non-biased representation of the mRNA population for each macrophage subpopulation.

3.4.3 Screening of cDNA library and isolation of goldfish granulin transcripts

Non-amplified PKM proliferative and senescence phase cDNA libraries were screened using standard procedures. Following the tertiary PCR-based screen, individual clones were PCR-amplified, confirmed to encode for a single-sized insert, and individually stored at 4°C in 500 μL of SM buffer and chloroform. Briefly, the unamplified PKM proliferative phase cDNA library was plated on NZY agar plates and plaque lifts were performed using nylon membranes (NEN Research). For each plate, duplicate lifts were made. Differential cross-screening of the library was performed using a [^{32}P]-labelled proliferative phase cDNA population as a probe to screen the first set of lifts and a [^{32}P]-labelled senescence phase cDNA population as a control probe to screen the second set of lifts. Mixed cDNA probes from both phases of PKM growth were prepared separately. Briefly, polyA RNA from proliferative and senescence phase preparations were purified independently as described above. Double-stranded cDNA was synthesized from 1 mg of polyA RNA using the Timesaver cDNA synthesis kit (Amersham Pharmacia) and used, unamplified, to ensure the most accurate representation of mRNA populations in each of the phases. For the primary and secondary differential screens, total cDNA populations were triple-labelled (a-^{32}P-dATP,a-^{32}P-dCTP, a-^{32}P-dGTP) using Prime-It II Random Primer Labelling kit (Stratagene) and radiolabeled cDNA was purified using STE Select-D G-50 spin columns (50 –30). Membranes were pre-hybridized at 65°C in Hybrizol II (Oncor) for at least 3 hours prior to the addition of denatured probe and subsequently hybridized overnight at 65°C in a hybridization oven. Following washing under stringent conditions (final wash with 0.1 SSC/0.1% SDS for 10 minutes at 65°C) the membranes were exposed to Kodak X-OMAT film and stored at -80°C for 1.5–36 hours. Autoradiographs from the proliferative phase cDNA-probed membranes were compared to the senescence phase cDNA-probed membranes. Signals that showed different levels of intensity were isolated as plaques were stored at 4°C in 500 mL of SM buffer + chloroform, and represented phage stocks of cloned transcripts that were differentially expressed between the proliferative and senescence phases.

3.4.4 *Production of goldfish macrophage cDNA library using suppressive subtractive hybridization (SSH)*

Goldfish macrophages isolated from 20 individual fish were pooled and 2.5 x 10^7 cells were stimulated with MAF and LPS (5 µg/mL) or were incubated in medium alone (un-stimulated) in separate tubes for 14 hours at 20°C. Messenger RNA was isolated (Oligotex kit, Qiagen, Valencia, CA) from total RNA (TriZol, Invitrogen) of control and stimulated macrophages. Messenger RNA from control (un-stimulated) and stimulated macrophages were subjected to SSH and selective PCR using the PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol, which includes an RsaI digestion step to reduce size bias. Differentially expressed cDNA fragments were sub-cloned into the pCR2.1-TOPO® vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen). Approximately 300 randomly-selected clones were then checked for homologies in the GenBank database using NCBI's BLASTX sequence comparison software at the NCBI website (www.ncbi.nlm.nih.gov/BLAST).

3.4.5 *RNA isolation, cDNA synthesis, RT-PCR and cloning*

Total RNA was isolated from perfused tissues and cells using Trizol (Invitrogen) following the manufacturers protocol. Three micrograms of total RNA was subsequently used to generate cDNA using the Superscript II cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Prior to PCR the cDNA templates were quantified and normalized to assure the same amount of cDNA was used in each PCR reaction. PCR amplification of the target transcript was performed as follows: 5 μL of the cDNA template was added to 76 μL ddH2O, dNTPs (0.2 μL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10X PCR buffer (10 μL of 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), primers (2.4 μL of each 20 μM solution) and a 15:1 ratio of Taq:Pfu DNA polymerases (1 μL of 5 U/L). Potential positive bands resulting from agarose electrophoresis (0.8-1.2% agarose gels) were excised from the gel and purified using the QiaQuick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. Purified PCR amplicons were then cloned into the PCR 2.1 TOPO TA vector using approximately 2 μL of PCR product and incubated for 15 minutes at room temperature with 0.5 μL of vector and 0.5 μL of salt solution provided with the vector by manufacturers. The mixture was then added to Top10 Competent cells (Invitrogen) and incubated on ice for 10 minutes. The Top10 cells were heat shocked at 42ºC for 30 seconds and then placed on ice for 1 minute. After 1 hour of shaking at 37ºC the cells were plated on agar plates containing 100 μg/mL Kanamycin and incubated at 37ºC overnight. The following day, colonies were identified and picked either for restriction digestion or colony PCR confirmation of insert presence and size.

3.4.6 *DNA sequencing and in silico analysis*

PCR-amplified clone inserts, corresponding to each of the confirmed positive clones, were purified using the QIAquick PCR purification kit (Qiagen), and sequenced using a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia) and a PE Applied Biosystems 377 automated sequencer v1.83. Sequence manipulations were performed using the Genetool (Biotools Inc. Edmonton, Canada) software package.

Sequence alignments were performed using CLUSTAL W Version 1.81. NCBI BLAST searches were performed for sequence comparison and identification of conserved homologous regions. Pfam (http://pfam.wustl.edu/hmmsearch.shtml) was used for identification of conserved protein motifs. Phylogenetic tree construction was performed using NJ Plot (http://pbil.univ-lyon1.fr/software/njplot.html). Signal peptides, trans-membrane regions, and molecular weights were predicted using Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html).

3.4.7 Quantitative PCR

Real-time PCR analysis was carried out using the Applied Biosystems 7500 fast real-time PCR equipment. The relative expression of transcripts in relation to endogenous controls of β-actin or GAPDH was assessed using primers generated with the Primer Express software (Applied Biosystems). Analysis of the relative tissue expression data was carried out using the 7500 Fast software (Applied Biosystems). Complementary DNA used in qPCR experiments was isolated and converted from total RNA as described in section 3.3.4 of this chapter. Tissues used and the PCR protocols designed and used varied for each target transcript.

Statistical analysis was performed by normalizing all data to the endogenous control and then setting one experimental value as 1. Expression differences between other experimental values and the treatment set to 1 were assessed by one-way ANOVA analysis, comparing the change in relative expression between the two samples.

3.4.8 *Isotopic Northern blot analysis*

Twenty five µg of total RNA was subjected to electrophoresis on a 1.5% agarose, 20% formaldehyde gel and transferred overnight to Gene screen Plus nylon membranes (NEN Research). Blots were screened using 200 ng of a probe created using RT-PCR. The probe was singly labelled using α-^{32}P-dCTP in combination with dATP, dGTP and dTTP and 0.5 µL of Klenow fragment over 4 hours at room temperature. The probe was purified using QIAquick gel extraction columns (Qiagen) using the manufacturer's protocol. Hybridization with the probe was allowed to proceed overnight at 42°C, and then washed 3 times with 2X SSC, 0.1% SDS for 5 minutes each, and 0.1X SSC, 0.1% SDS 3 times for 20 minutes. Blots were then exposed to Kodak X-OMAT film and stored at -80°C for 24 hours before they were developed.

3.4.9 *Non-isotopic Northern blot analysis*

PCR amplicons generated using primers spanning a 500bp area of the target transcript were ligated into the pCR 2.1 TOPO vector (Invitrogen) following the manufacturer's protocol. Sense and anti-sense RNA probes of the target were synthesized using T7 RNA polymerase (Invitrogen) to drive RNA synthesis using the T7 promoter of the pCR 2.1 TOPO vector. Briefly, the vector was linearized using the restriction enzyme Kpn1 (Fermentas). Linearized vector was phenol/chloroform extracted and re-suspended in RNAse free water. Linearization was confirmed by running a sample of the purified DNA on an agarose gel. Sense and anti-sense probes were synthesized by adding 2 µg of linearized DNA to a reaction containing T7 RNA polymerase and a DIG label in the recommended buffers supplied with the DIG label

(Roche). RNA probe synthesis was allowed to continue for 2 hours, after which the probe was purified using Sigma Spin post-reaction purification columns (Sigma) according to the manufacturer's protocol. The presence of a purified probe was confirmed using a 2.2 M formaldehyde gel and, after quantification, the probe was stored at -80°C.

After probe construction, 25 μg of total RNA was subjected to electrophoresis on a 1.5% agarose, 20% formaldehyde gel and transferred overnight to Gene screen Plus nylon membranes (NEN Research). Two hundred nanograms of the anti-sense probe was incubated with the blot overnight at room temperature and then the blot was washed 3 times with 2X SSC, 0.1% SDS for 5 minutes each, and 0.1X SSC, 0.1% SDS 3 times for 20 minutes and then finally 3 times for 5 minutes in TBS. After washing, the blot was incubated for 1 hour in mouse anti-DIG antibody conjugated with alkaline phosphatase. After incubation, the blot was washed 3 times with TTBS for 10 minutes, and then 3 times in TBS for 15 minutes. After washing, the blot was developed using BCIP and NBT (Roche) according to the manufacturer's protocols.

3.4.10 Analysis of the zebrafish genome

Genomic sequences were obtained for two putative zebrafish M17 transcripts (Genbank Accession Numbers: XM679703 and XM678310) by BLASTx analysis of the zebrafish genome using the facilities at the NCBI webpage (http://www.ncbi.nlm.nih.gov/). Regions of genomic sequence that flanked the predicted zebrafish M17 genes were also assessed using NCBI and were compared to human and mouse genomic sequences using BLAST human and mouse genome, respectively.

Syntenic regions were identified by comparing the encoded genes located within all three genomic sequences.

3.4.11 Prokaryotic recombinant protein expression

PCR amplification of the targeted protein expression construct insert was performed as follows: 7 µL of the target clone template was added to 76 µL ddH2O, dNTPs (0.2 µL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10X PCR buffer (10 µL of 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), expression primers (2.4 µL of each 20 µM solution) and a 15:1 ratio of Taq:Pfu DNA polymerases (1 µL of 5 U/L solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient thermal cycler. Amplification was confirmed by agarose gel electrophoresis.

The target transcript amplicon was cloned into the pET SUMO TA or the pET 151/TOPO expression vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen) according to the manufacturer's specifications. Cells were plated onto LB-Ampicillin (100 µg/mL) or Kanamycin (100 µg/mL) plates and incubated overnight at 37°C. Positive clones were identified by randomly picking 10 colonies and using them as templates for PCR reactions using the vector specific primers T7 forward and reverse (Invitrogen). Positive clones were grown overnight in 5 mL of LB medium containing 100 µg/mL Ampicillin or Kanamycin and plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen). Once positive clones were isolated, restriction digests followed by gel electrophoresis verified the presence of insert and vector DNA. Plasmids were sequenced, as described above, in order to confirm that inserts were ligated into the expression vector in the proper orientation and in frame. Sequence data were analyzed using Genetool (Biotools).

3.4.12 Eukaryotic recombinant protein expression

Eukaryotic recombinant protein expression was conducted using either an insect-based protein expression system or a mammalian protein expression system. PCR amplification of the protein expression construct insert was performed as follows for both systems: 7 μL of target transcript template was added to 76 μL ddH_2O, dNTPs (0.2 μL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10X PCR buffer (10 μL of 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), expression primers (2.4 μL of each 20 μM solution) and a 15:1 ratio of *Pfu*:*Taq* DNA polymerases (1 μL of 5 U/L). PCR amplification was conducted in an Eppendorf Mastercycler Gradient thermal cycler. Amplification was confirmed by agarose gel electrophoresis.

The PCR-amplified transcript was cloned into the pIB/V5-His-TOPO expression vector (Invitrogen) for insect expression or the pSeqTag2 mammalian expression system (Invitrogen) for mammalian cell expression, and transformed into chemically competent TOP10 *E. coli* (Invitrogen) according to the manufacturer's specifications. Once positive clones were isolated, restriction digests followed by gel electrophoresis verified the presence of insert and vector DNA. Plasmids were sequenced, as described above, in order to confirm that inserts were ligated into the expression vector in the proper orientation and open reading frame. Sequence data was analyzed using Genetool (Biotools) and Vector NTI (Invitrogen) software packages.

3.4.12 Statistical analysis

Statistical analysis was done using a one-way ANOVA (analysis of variance). Differences calculated were deemed statistically significant if $P < 0.05$. Unless stated otherwise, all data was further analyzed using the Tukey A post-hoc test.

3.5 Production and purification of recombinant proteins

3.5.1 Production of recombinant prokaryotic

Plasmid DNA containing the target transcript expression constructs was transformed into BL21 Star™ (DE3) One Shot® *E. coli* (Invitrogen) for recombinant protein expression. 10 ng of plasmid DNA was transformed into the bacteria which was then grown overnight at 37°C in LB medium containing 50 µg/mL kanamycin. Induction of recombinant protein expression was performed in a pilot expression experiment by the addition of IPTG according to the manufacturer's protocols. Briefly, 10 mL of LB medium containing 50 µg/mL kanamycin was inoculated with 500 µL of an overnight culture (described above) and allowed to grow for 2 hours at 37°C with shaking until they reached mid-log phase of growth (i.e. O.D.600=0.5–0.8). IPTG was then added to a final concentration of 0.75 mM and a 500 µL aliquot was removed from the culture, and centrifuged at 10,000 × *g* using a microcentrifuge for 30 seconds. The supernatants were removed and the cell pellets frozen at −20°C (these were the 0 hour time-point samples). Remaining cultures were incubated at 37°C with shaking and 500 µL aliquots removed after 1, 2, 4, and 6 hours post-induction. For each time-point, samples were processed as described above. Individual samples were then analyzed by SDS-PAGE and Western blot for the presence of recombinant protein expression.

For large-scale expression and purification of the target proteins, 50 mL of LB medium containing 100 µg/mL carbenicillin was grown overnight at 30°C with shaking to an O.D.600. Ten millilitres of this culture was then inoculated into 250 mL of LB (100 µg/mL carbenicillin) and a total of 4 flasks were prepared (1 L total). Cultures were incubated until mid-log phase of growth was achieved followed by the induction of target protein expression with 0.1mM IPTG. Cultures were then grown for 2 hours prior to the purification of the recombinant molecules.

3.5.2 Purification of recombinant prokaryotic proteins

Recombinant proteins expressed in the pET/SUMO and pET/151 vectors were engineered to contain a N-terminal 6xHis tag to facilitate subsequent purification and detection protocols. Bacteria were pelleted by centrifugation at 2000 x *g* and supernatants were collected. Proteins were purified from culture supernatants using MagneHIS beads (Promega) according to the manufacturer's specifications. Purified proteins were eluted in a solution containing 100 mM HEPES and 500 mM imidazole, and then dialyzed overnight against 1X PBS. Endotoxin was removed using polymixin B columns (Sigma) and then the samples were dialyzed again, overnight, against 1X PBS. After dialysis, LPS removal was confirmed using the Pyrogent LPS detection assay (Lonza). Protein samples were then filter-sterilized in preparation for immunodetection and analysis of biological activity. Total protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's protocols. The identities of all recombinant proteins were confirmed using mass spectroscopy.

3.5.3 *Production of recombinant proteins in the insect expression system and selection of stable cell lines*

Drosophila KC and Sf9 embryonic cells were grown in ESF-921 medium (Expression Systems) at 27°C. Sf9 cells were transferred into Grace's insect medium (Sigma), and transfected with 5 μg of expression plasmid using Cellfectin Reagent (Invitrogen) according to the manufacturer's instructions. Stable transfectants were selected with blasticidin at 75 μg/mL. After two rounds of selection, the stable cell lines were maintained in ESF-921 medium containing 10 μg/mL blasticidin for 5-6 days at 27°C prior to sub-culturing and collection of supernatants containing recombinant soluble CSF-1R.

3.5.4 *Purification of recombinant proteins produced in insect cells*

Recombinant proteins expressed in the pIB/V5 HIS-TOPO vector were engineered to contain a C-terminal 6x His tag and V5 epitope to facilitate subsequent purification and detection protocols. Stably transfected Sf9/KC cell line supernatants were dialyzed against Ni-NTA wash buffer and concentrated in preparation for purification. Recombinant protein was purified using Ni-NTA agarose columns (Qiagen) according to the manufacturer's specifications. Purified proteins were eluted in Ni-NTA wash buffer, dialyzed extensively against 1X D-PBS, and filter-sterilized in preparation for immunodetection and analysis of biological activity. Total protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's protocols.

3.5.5 *Production of recombinant proteins in mammalian cells*

Plasmid DNA containing the expression constructs was transformed into Chinese hamster ovary (CHO) cells (Invitrogen) for recombinant protein expression. Ten nanograms of plasmid DNA was transfected into CHO cells using 293fectin (Invitrogen) according to the manufacturers specifications. After transfection, cells were transferred to a 25 cm^2 culture flask and incubated for 2 days before supernatants were collected and tested for recombinant protein by Western blot using an antibody specific for the 6x HIS tag located on the C-terminal end of the recombinant protein. Cultures that were successfully transfected were placed in a 2 L culture roller bottle and grown for 7 days at 37°C after which the supernatants were collected.

3.5.6 *Purification of recombinant proteins produced in mammalian cells*

Supernatants collected from the transfected CHO cells were collected and dialyzed overnight against 1X PBS and pooled together. Recombinant proteins were purified from dialyzed culture supernatants using MagneHIS beads (Promega) according to the manufacturer's specifications. Purified proteins were eluted in a solution containing 100 mM HEPES and 500 mM imidazole (pH 9.0), and then dialyzed overnight against 1X PBS. Samples were then filter-sterilized in preparation for immunodetection and analysis of biological activity.

3.5.7 *Confirmation of peptide identity by mass spectroscopy*

The identities of the recombinant proteins was confirmed by reverse-phase highpressure liquid chromatography and tandem mass spectrometers (LC/MS/MS) at the Institute of Bio-molecular Design, Medical Sciences Building at the University of Alberta, Edmonton, Canada. The peptides were analyzed by MASCOT (Matrix Science) protein search engine.

3.6 Immunodetection of recombinant proteins

3.6.1 Immunodetection of recombinant granulin

Purified recombinant proteins were detected by Western blot analysis using the N-terminal HIS tag on the recombinant protein (using an anti-6x HIS mAb; Invitrogen), or with anti-V5 antibody specific to the V5 epitope produced in some vectors. Briefly, proteins were separated by SDS-PAGE under reducing conditions using 12.5% polyacrylamide gels, transferred to 0.2 μm nitrocellulose membranes (BioRad), and incubated over night at 4°C in the presence of the primary antibody. Membranes were subsequently washed, incubated with an horseradish peroxidase-conjugated mAb, and developed using the ECL Advance™ Western Blotting Detection Kit (Amersham Biosciences) according to the manufacturer's specifications. In other cases, after washing, the membranes were incubated with an alkaline phosphatase (AP)-conjugated mouse IgG mAb, and developed using BCIP and NBT (BioRad) according to the manufacturer's protocol.

3.6.2 Production and purification of rabbit polyclonal antibodies against recombinant proteins

Recombinant goldfish granulin, M17 (goldfish LIF), or sCSF-1R were used to immunize rabbits. The primary immunization was performed by combining an equal volume of purified recombinant protein (100 μg) in 750 μL of Freund's Complete Adjuvant. Booster injections were done exactly as the primary immunizations except that Freund's Incomplete Adjuvant was used.

The IgG fraction was purified by precipitation using saturated ammonium sulphate, solubilization of precipitate in PBS, and purification using a HiTrap Protein A HP column (Amersham) according to the manufacturer's protocol. Fractions containing IgG were pooled and filter-sterilized (0.22 μm filter; Millipore). Specificity of the antibody was determined by reactivity with purified recombinant granulin using Western blot analysis under native and denaturing conditions.

3.7 Detection of native proteins and protein interactions

3.7.1 Far Western blot of native CSF-1

Native goldfish CSF-1 was initially detected by probing a Western blot with the recombinant goldfish soluble CSF-1R. Goldfish culture supernatants were passed through an Affi-gel Blue column (BioRad) to remove albumin from the samples. After removal of albumin, the supernatants were concentrated 10X and dialyzed against 1X PBS. The concentrated supernatants were then run on a 12% SDS-PAGE and transferred to nitrocellulose membrane. The blot was then incubated in 100 ng/mL of recombinant soluble CSF-1R overnight and then washed 3 times in TTBS. The blot was then incubated in anti-sCSF-1R antibody overnight and washed 3 times in TTBS. After washing, the blot was incubated in an anti-rabbit polyclonal antibody conjugated to alkaline phosphatase for 1 hour. After incubation, the blot was washed 3 times in TTBS and 3 times in TBS before being developed using BCIP and NBT (BioRad) according to the manufacturer's specifications.

3.7.2 Detection of native granulin in primary kidney macrophage supernatants

Native goldfish granulin was detected in 4X concentrated goldfish PKM supernatants obtained from day 5 PKM cultures. Goldfish culture supernatants were passed through an Affi-gel Blue column (BioRad) to remove albumin from the samples. After removal of albumin, the supernatants were concentrated 4X, and dialyzed against 1X PBS. The concentrated supernatants were then run on a 12% SDS-PAGE and transferred to nitrocellulose membrane. Western blots of the concentrated samples were probed overnight using the anti-goldfish granulin antibody (1:1000) and then washed 3 times using TTBS the next day. After washing, the membranes were incubated for 1 hour with a horseradish peroxidase-conjugated mAb. After incubation, the membranes were washed 3 times with TTBS and 3 times with TBS and then developed using the ECL Advance™ Western Blotting Detection Kit (Amersham Biosciences) according to the manufacturer's specifications.

3.7.3 Goldfish rgCSF-1/sCSF-1R binding assay

Goldfish rgCSF-1 and soluble CSF-1R were biotinylated using the EZ-Link Biotinylation Kit (Pierce) according to the manufacturer's protocol. Effective biotinylation of each protein was confirmed using the EZ-Biotin Quantitation kit (Pierce) according to the manufacturer's protocol. To determine whether rgCSF-1 formed a homodimer before interacting with its receptor and to see if rgCSF-1 and the sCSF-1R interacted *in vitro,* 100 μg of each protein was incubated in de-ionized water (pH 7.5) for 1 hour. The protein mixture was then cross-linked using bis (sulfosuccinimidyl) suberate (BS^3) for 15 minutes and then run on a reducing SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane (Bio-Rad) and probed using HRP-conjugated avidin (Pierce). The blot was then developed using ECL (Pierce) on x-ray film (Kodak). To confirm that cross-linking of CSF-1 was not an artefact of non-specific protein interactions 100 μg of CSF-1 was incubated with 100 μg of purified BSA (Roche) and cross-linked using the above protocol.

3.8 *In situ* hybridization and gene knockdown experiments

3.8.1 In Situ Hybridization

DIG-labelled probed were synthesized as mention above in section 3.3.7 of this chapter. Whole-mount *in situ* hybridization was carried out on various developmental stages of zebrafish as described previously [31, 419]. For RNA *in situ* hybridization, embryos were treated with proteinase K and hybridized at 65°C with DIG-labelled probes. Hybridization of probes was detected with anti-digoxigenin (Roche) and NBT/BCIP (Roche).

Embryo preparations were manually de-yolked, mounted in glycerol and viewed on a Leica DMRXA upright microscope equipped with epifluorescence (10X and 20X objective). Image capture was done using an Optronics camera and the Picture frame software. Figures were assembled using Adobe Photoshop CS.

3.8.3 *Generation and analysis of zebrafish morpholinos*

Morpholino oligonucleotides (MOs) towards zebrafish *lif* and *lifr* were selected based on the 5' un-translated region and open reading frame of the zebrafish *lif* and *lifr* sequences [420]. Translation-blocking *lif* and *lifr* morpholinos were generated by Gene-tools (www.gene-tools.com).

Morpholinos were injected into single cells stage zebrafish embryos at concentrations of 2.5 mg/mL, in volumes of approximately 5 nL. Injected and non-injected embryos were then incubated in embryo medium at 28.5°C for 24 hours, after which they were assessed for viability and changed to embryo media containing PTU to prevent pigment formation. Wild type and Isl1-GFP WIK transgenic zebrafish embryos injected with *lif* and *lifr* morpholinos were assessed for morphological differences from non-injected controls under an Olympus SZX12 stereoscope at 24, 48 and 72 hours hpf. Isl1-GFP transgenic zebrafish were stage matched to 48 hpf and fixed in 4% paraformaldehyde for 4 hours and then analyzed for differences in branchiomotor neuron development and migration from non-injected controls using a Zeiss AxioImager Z1 compound microscope. Z-stack images were photographed using a Zeiss LSM 510 confocal microscope at 488 nm under a 20X objective, and were compiled using Zeiss LSM Image Browser software.

3.8.4 *Rescue of lifr morphant phenotype*

Rescue of the *lifr* morpholino-induced phenotype was done by injecting synthetic *lifr* mRNA into the single cell embryo prior to injection of the *lifr* morpholino. Due to lack of 5' UTR sequence information, a portion of the morpholino was created to the *lifr*

ORF. This required the introduction of silent mutations to the 5' ORF of the *lifr* mRNA construct to prevent binding of the morpholino to the rescue mRNA. This PCR product was cloned into the T7TS expression vector [421] and transformed into Top 10 competent cells (Invitrogen) before being grown overnight at 37ºC, in 100 mL of LB medium containing 50 μg/mL Ampicillin. Bacteria were collected and maxi-prepped using the QiaQuick Maxi Prep kit (Qiagen) according to the manufacturer's protocol. The plasmid containing the *lifr* clone was then quantitated and 10 μg was linearized. *lifr* mRNA was synthesized off of the linearized plasmid using T7 RNA polymerase (Invitrogen). The synthetic mRNA was quantitated and diluted in DEPC water to a concentration of 0.1 μg/μL for injection into the embryo. Rescued embryos were counted and visualized using a Zeiss LSM 510 confocal microscope.

3.8.5 Analysis of acetylated tubulin expression in embryonic zebrafish

Embryonic axon development was visualized in non-injected *lif* and *lifr* morpholino-injected embryos using an anti-acetylated tubulin antibody (Sigma-Aldritch). Briefly, embryos were fixed at 48 hpf in Dents fixative (80% methanol, 20% DMSO) overnight at 4°C and then washed 3 times for 10 minutes in PBS-T (0.5% Tween-20). Embryos were then blocked for one hour in PBS-T with 2% BSA and 10% goat serum and incubated overnight at 4°C in anti-acetylated tubulin monoclonal antibody (Sigma-Aldritch) at a 1:500 dilution. Embryos were washed the next day 3 times for 10 minutes in PBS-T and then blocked for 1 hour and incubated in a goat anti-mouse secondary antibody conjugated to Alexa fluor 488 (1:1000; Molecular Probes). Embryos were de-yolked, cleared in 70% glycerol, mounted, and photographed using a Zeiss LSM 510 confocal microscope under a 20X objective.

3.8.6 RNA interference in goldfish primary kidney macrophages

RNAi transfection procedures were optimized using the Alexafluor (Invitrogen) transfection control oligo in combination with 3 different liposomal transfection reagents; Oligofectamine (Invitrogen), Cellfectin (Invitrogen) and 293fectin (Invitrogen). For the optimization experiment, PKM macrophage cultures from 6 fish were polled together, counted and seeded in 6-well plates at a concentration of 1 x 10^6 cells/well in 1 mL of complete medium and incubated at 23°C overnight. A mixture of either 5 μL or 10 μL of transfection reagent with 10, 50 or 100 nM concentrations of the Alexafluor oligo was prepared in 1.5 mL tubes and incubated at for 30 minutes at room temperature. The cell cultures were centrifuged for 10 minutes at 300 x *g*, the supernatants removed and the cells re-suspended in 200 μL of Alexafluor mixture and 800 μL of incomplete medium and incubated for 5 hours at 23°C. After incubation, 500 μL of 2X complete medium was added to each well. The cells were incubated 23°C for 18 hours after which they were centrifuged at 300 x *g* for 10 minutes and the cells were re-suspended in 1 mL of complete medium. The cells were washed 3 times in 1 mL of complete medium. After washing, the cells were dislodged from the well by gentle pipetting and then 20 μL of the cell suspension was placed on a microscope slide. Cells were counted in 20 random fields of view (100X objective) and the total number of cells and the number of red fluorescing cells determined. The mean number of fluorescing cells was compared to the mean number of total cells to determine the most effective transfection method for the goldfish macrophages. Ten microlitres of 293fectin was found to be the most effective procedure for transfecting the cells.

3.8.7 Confirmation of RNAi-induced knockdown of CSF-1R mRNA

RNAi-induced knockdown of the CSF-1R mRNA transcript was confirmed by non-isotopic Northern blot analysis. A CSF-1R-specific probe was synthesized and the PCR amplicon generated by this primer pair was then cloned into the PCR 2.1 TA vector (Invitrogen) following the manufacturers protocols. The clone was sequenced using the protocol stated above, to confirm that the insert was cloned in the correct direction and contained no mutations. T7 RNA polymerase was used to drive transcription of the insert and thereby synthesize an anti-sense RNA oligo specific for the CSF-1R. The oligo was purified and quantitated as mentioned above and then incubated with DIG in order to label the probe. RNA isolated from cells incubated with 1, 2.5, 5, 7.5, 10 or 15 nM RNAi oligos was run in a 1% agarose gel at a concentration of 30 ng RNA per well. The RNA was then transferred to nitrocellulose membrane (BioRad) and cross-linked under UV light. The membrane was then washed 3 times and then incubated with the anti-CSF-1R probe overnight. The following day, the blot was washed 6 times and then incubated with an AP-conjugated anti-DIG antibody for 1 hour. The blot was then washed again 6 times and developed using BCIP and NBT (BioRad) until bands were observed.

3.9 Generation of long-term goldfish macrophage cultures from kidney and blood using recombinant goldfish CSF-1

Effective concentrations of recombinant goldfish CSF-1 (10 ng/mL) was used to determine whether it supported long-term growth of goldfish macrophages. Cells were isolated as described and incubated for 2 days. On day 2 post-isolation the cells were re-suspended and centrifuged at 300 x *g* for 10 minutes. After centrifugation, the supernatants were removed and the cells re-suspended in complete medium without CCM. To each culture flask, 10 ng/mL rgCSF-1 was added, and the cultures incubated at 20ºC. Cell cultures were monitored every two days and were assayed using flow cytometry every 10 days to determine the cell subpopulations present. When cultures became confluent, the cells were sub-cultured using half of the original culture to seed the new cultures.

3.9.1 Analysis of long-term primary kidney macrophage/primary blood monocyte functions

Functional properties of the long-term kidney and blood leukocyte cultures was assessed by first analyzing their morphological properties using confocal and DIC imaging in addition to observing their flow cytometric profiles based on size and internal complexity. After morphological characterization, the cells were assayed for their ability to produce antimicrobial responses to macrophage activation factor (MAF) and MAF with LPS. Both nitric oxide production and reactive oxygen intermediate production were assessed using the protocols described above. Finally, the proliferative response of the cells following the addition of recombinant goldfish granulin, LIF and/or CSF-1 were measured using the BrdU proliferation assay described earlier in this chapter.

Table 3.1 Table of all primers and oligonucleotides used in this thesis research.

Primer Name	Sequence
PCR primers	
Goldfish LIFR S	AGGGCAAGCAGTGGTATCAACGC
Goldfish LIFR AS	AAACCGTGGACCAGAACCTAGA
Zebrafish *lif* S	CTGCTGATGCTGAGGATGAGGATGAT
Zebrafish *lif* AS	GACGATGCATCCATAGACCTTCTGCT
Zebrafish *lifr* S	GCAACAGGACCTACATCACAGAGA
Zebrafish *lifr* AS	CCCATGGCTTTGTTGATGAGACAG
Zebrafish actin S	GCACGCGACTGACACTGAAG
Zebrafish actin AS	GAAGGCCGCTCCGAGGTA
Quantitative PCR primers	
Goldfish granulin S Quantitative	TTGATGTTACTCATGGCAGCTCTT
Goldfish granulin AS Quantitative	GGGCCTGAGAGATCCATCATT
Goldfish actin S Quantitative	GCACGCGACTGACACTGAAG
Goldfish actin AS Quantitative	GAAGGCCGCTCCGAGGTA
Goldfish LIF S Quantitative	ATGGTCTGCCTGTCTCAGAGA
Goldfish LIF AS Quantitative	TACACGTCTTTTTCATGGTTTT
Goldfish CSF-1 S Quantitative	ATGAACACACACATAACAGCCCACAA
Goldfish CSF-1 AS Quantitative	AGGATGAAGCACTGATGCCTTACCT
Recombinant expression primers	
Goldfish granulin S Prokaryotic expression	CACCCTCATGGCAGCTCTTGTAG
Goldfish granulin AS Prokaryotic expression	ACGGGGGTTGTTTACTTAC
Goldfish LIF S Prokaryotic expression	AGCTGCAGCCAGCTTCTTC
Goldfish LIF AS Prokaryotic expression	AGAACCATCTTTGGTAGTT
Goldfish sCSF-1R S Insect expression	GCTATGGCCTTTGCTCTCCTGTTCGTCTGT
Goldfish sCSF-1R AS Insect expression	ATGAAACTCACGCTGAATGACG
Goldfish CSF-1 S Mammalian expression	ATGAACACACACATAACAGC
Goldfish CSF-1 AS Mammalian expression	GATGATGTCACTTGATATACAG
Zebrafish morpholino oligonucleotides	
Zebrafish *lif*- specific Morpholino	CAATCTCTGAGACAGGCAGAGCATG
Zebrafish *lifr*- specific Morpholino	AGCACTCAATAGCCAGACCGACATG
Zebrafish *lifr* S mRNA rescue construct	AGATCTATGAGCGTATGGTTGCTCTCTGCTTTGCTTGTGTCTGATTGAGC
Zebrafish *lifr* AS mRNA rescue construct	TGATCATGTGCGTGGTTGTGCAAACTTAC
Goldfish CSF-1R RNAi oligonucleotides	
Goldfish CSF-1R S RNAi Probe	ATGTTTGCTCTCCTGTTCGT
Goldfish CSF-1R AS RNAi Probe	GTTTTCTGGGCAGGTGGTTC
Goldfish CSF-1R dsRNA duplex 1	GUCGAGUCCCUGUGCCACCUGAUGGGA
Goldfish CSF-1R dsRNA duplex 2	CUGAAAUCUCUCUCCCACCAGUUUGAC

Chapter 4

Molecular and Functional Analysis of Goldfish Macrophage Colony Stimulating Factor[1]

4.1 Introduction

Colony stimulating factor-1 (CSF-1 = M-CSF) is the primary growth factor involved in the regulation of survival, proliferation and differentiation of mononuclear phagocytes and their precursors [422]. CSF-1 has also been demonstrated to play important roles in bone metabolism, atherogenesis, inflammation, pregnancy and in pre-implantation development of the female reproductive tract [10, 150, 423]. By alternative splicing, three mature peptides can be formed [133]; a secreted soluble glycoprotein that acts humorally, a cell membrane anchored glycoprotein, and an extracellular matrix anchored proteoglycan that act in a paracrine manner [133, 424-429]. All forms of CSF-1 initiate their effects by a high affinity interaction between the CSF-1 molecule, which is commonly active as a homodimer, and the CSF-1 receptor (CSF-1R) which is a member of the class III receptor tyrosine kinase family [179, 430-432].

From an evolutionary perspective, macrophages and their functions are quite conserved: macrophage-like cells can be found in almost all multi-cellular organisms. Macrophage development is relatively unknown in a majority of these organisms with the exception of mammals. In contrast to the mammalian primary macrophages that require addition of exogenous growth factors (commonly CSF-1) for their growth [179, 433], bony fish macrophages secrete their own growth factors and exhibit spontaneous growth *in vitro* in the absence of exogenous growth factors [24, 44, 46]. Until recently, mammals were the only group of organisms in which CSF-1 had been identified. That the CSF-1-regulated pathway of macrophage development exists in organisms other than mammals was suggested by recent evidence showing the presence of CSF-1 transcripts in the chicken [238, 239] and bony fish [24, 44, 52], as well as the identification of CSF-1R in the goldfish [49], puffer fish [434], rainbow trout [242], zebrafish [435] and the gilthead sea bream [241].

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[1] A version of this chapter has been published:
Hanington et al. 2007. Growth factors of lower vertebrates: characterization of goldfish (*Carassius auratus* L.) macrophage colony stimulating factor. Journal of Biological Chemistry. 282: 31865-72.

In addition to CSF-1's role as a growth factor it has also been implicated in a number of different macrophage activation processes such as induction of microbicidal activity [173, 436], up regulation of activation-associated cytokines [3], increased chemotactic and chemokinetic activity leading to recruitment of monocytes/macrophages to an infection or wound site [167, 437, 438], and enhancement of phagocytosis in response to pathogens [166, 171, 439, 440].

Besides being capable of activating macrophages, CSF-1 also cooperates with a number of cytokines to further enhance host defence [441]. For example, it has been shown that CSF-1 acts in conjunction with tumour necrosis factor alpha (TNF-α) to synergistically stimulate progenitor cell proliferation and to enhance respiratory burst and chemotactic activity [441-443].

In this chapter, I describe the identification and biological characterization of the first CSF-1 from a non-mammalian organism, the goldfish.

4.2 Experimental design

4.2.1 *Quantitative PCR analysis of CSF-1 expression in different tissues*

The relative mRNA levels of goldfish CSF-1 in the kidney, spleen, liver, heart, gill, intestine, and brain, as well as in sorted PKM progenitor cells, monocytes and macrophages were determined by quantitative PCR, using procedures described in chapter 3.

4.2.2 *Expression of a soluble CSF-1 receptor expression constructs for recombinant protein expression in Sf9 insect cells*

The sCSF-1R was expressed using an insect-based protein expression system. The PCR-amplified sCSF-1R insert was cloned into the pIB/V5-His-TOPO expression vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen). The recombinant protein was produced in Sf9 lepidopteran embryonic cells as described in chapter 3.

4.2.3 *Expression of CSF-1 using Chinese hamster ovary (CHO) cells*

Goldfish CSF-1 transcripts were designed for recombinant expression in the pSeqTag2 mammalian expression system (Invitrogen). The plasmid was chemically transfected into CHO cells as described in chapter 3.

4.2.4 *Macrophage proliferation induced by purified recombinant colony stimulating factor-1 (rgCSF-1)*

The effect of goldfish rgCSF-1 on macrophage proliferation was assessed using the cell proliferation ELISA BrdU colorimetric assay (Roche). Macrophages were grown as described in chapter 3 and sorted using a FACS Calibur flow cytometer (Becton Dickinson) based on cell size and cellular complexity. Progenitor cells, monocytes and macrophages were counted and seeded at a density of 1x 10^4 cells/well in 96-well culture plates (Falcon) in 50 μL of incomplete medium. Duplicate treatments of cell cultures from 8 fish (n=8) were treated with 50 ng/mL, 25 ng/mL, 10 ng/mL, 5 ng/mL and 1 ng/mL of rgCSF-1 resuspended in 50 μL of incomplete cell culture medium, and incubated with 15 μM of BrdU labelling reagent. Treatments were re-applied on days 1, 3, 5, and 7 on alternate days that samples were collected. Blocking studies were done using the same rgCSF-1 concentrations in 50 μL of medium and the following: 10 ng/mL, 50 ng/mL, 100 ng/mL or 250 ng/mL anti-CSF-1R affinity-purified rabbit IgG, or 10 ng/mL, 25 ng/mL, or 50 ng/mL sCSF-1R dissolved in 50 μL of medium. Pre-incubation with the anti-CSF-1R antibody or the sCSF-1R was done for 12 hours at 4°C. Cells were incubated with BrdU labelling reagent for 24 hours, and the test samples were taken every 2 days from the day 0 time point. The reaction was developed according to the manufacturer's specifications and optical densities determined at 450 nm using a microplate reader (Biotek). The colorimetric reaction was found to be directly proportional to the number of proliferating macrophages in culture. Recordings from the non-treated cells were subtracted from the experimental groups to account for the endogenous production of growth factors by goldfish macrophages.

4.2.5 *Nitric oxide induction by goldfish recombinant TNF-α (rTNF- α) and rgCSF-1*

Induction of a nitric oxide (NO) response in goldfish macrophages by rgCSF-1 was determined by seeding in triplicate 5 x 10^4 cells/well from 5 fish (n=5) in 50 μL of medium in 96 well plates (Costar). Macrophages were then treated with 50 μL of 1 ng/mL, 5 ng/mL, or 10 ng/mL of rgCSF-1, 100 μg/mL recombinant goldfish TNF-α (rTNF-α), 15 μg/mL or 100 μg/mL of rTNF-α in combination with 10 ng/mL rgCSF-1 dissolved in 50 μL of medium. Treated cells were incubated for 72 hours before the nitrite accumulated in the cultures was measured using the Griess reaction as described in chapter 3.

4.2.6 *Reactive oxygen intermediate production induced by goldfish rgCSF-1 and rTNF-α*

Four to 6 day PKMs from 5 fish were seeded in duplicate into 96 well plates at a density of 3 x 10^5 cells per/well and treated with either 100 ng/mL of rTNF-α, 1 ng/mL, 5 ng/mL, or 10 ng/mL of rgCSF-1, or a combination of 100 ng/mL rTNF-α and 10 ng/mL rgCSF-1 diluted in 50 μL of medium for 5 or 18 hours. Reactive oxygen intermediate production was measured using the NBT reduction assay described in chapter 3.

4.2.7 *Chemotaxis of monocytes/macrophages induced by goldfish rgCSF-1 and rTNF-α*

Treatments of rgCSF-1 (1 ng/mL, 5 ng/mL, or 10 ng/mL), rTNF-α (100 ng/mL) and a combination of rgCSF-1 (10 ng/mL) and rTNF-α (100 ng/mL) were applied to the lower wells of a leucite chemotaxis chamber (Nucleoprobe Corp.) and the chamber overlaid with polycarbonate membranes (5 µm pore size; Nucleopore Corp.). The chemotaxis assay was done in duplicate as described in chapter 3 using macrophages from 4 fish (n=4). Cells that had migrated to the underside of each membrane were counted by randomly selecting 20 fields of view and counting the cells under the 100X objective. The average number of cells that had migrated on each membrane was calculated from these 20 random counts and then a total average out of the 4 fish in each treatment was used to calculate an average for each treatment.

4.2.8 Phagocytosis of primary kidney macrophages induced by rgCSF-1 and rTNF-α

Four to 6 day PKMs from separate cultures established from 4 fish (n=4) were seeded in duplicate into 5 mL polypropylene tubes at a density of 3 x 10^5 cells/well. Cell cultures were treated with either 100 ng/mL of rTNF-α, 1 ng/mL, 5 ng/mL, or 10 ng/mL of rgCSF-1, or a combination of 100 ng/mL of rTNF-α and 10 ng/mL of rgCSF-1, and to each tube, fluorescence beads (2.0 µm diameter YG, Polysciences) were added at a ratio of 10 beads to 1 macrophage in a final volume of 100 µL/well. The phagocytosis assay was performed as described in chapter 3.

4.2.9 Impact of RNAi mediated knockdown of CSF-1 receptor on proliferation and differentiation induced by rgCSF-1

Cellular proliferation in cultures of monocytes in which CSF-1R was knocked down was assessed by direct cell counts to ensure there was no interference from the

RNAi transfection with the BrdU assay. Cell cultures from 8 individual fish were isolated and cultured for 1 day (see chapter 3) before being counted and re-suspended to a concentration of 1 x 10^6 cells/mL in medium. Each PKM culture was then split into four 25cm^2 flasks and the following four experimental groups were established: non-treated cells or cells treated with 10 µL/mL of 293fectin (Invitrogen), 10 nM of CSF-1R specific dsRNA oligos, or 10 µL/mL of 293fectin pre-incubated for 20 minutes with 10 nM CSF-1R specific dsRNA oligos. Cell cultures were incubated at 20ºC for 18 hours and were then transferred to 96 well plates. Twenty thousand cells in 50 µL from each experimental group were placed in triplicate into each well of a 96 well plate and allowed to incubate for 1 hour. After incubation, each well was treated with either medium, CCM, 1 ng/mL, 5 ng/mL or 10 ng/mL of rgCSF-1, or 1 ng/mL, 5 ng/mL, or 10 ng/mL of rgCSF-1 pre-incubated with 10 ng/mL, 25 ng/mL or 50 ng/mL of sCSF-1R, diluted in 50 µL of medium. Day 0 time points were incubated for 1 hour and then centrifuged at 300 x *g* for 10 minutes, after which the supernatants were removed and the cells were re-suspended in 50 µL of Trypsin-EDTA solution to detach adhered cells. After 5 minutes in the Trypsin-EDTA solution, 50 µL of complete medium was added to neutralize the trypsin and the cells were counted using a haemocytometer. The same procedure was used to count cells on days 2, 4, 6 and 8 post treatment. To confirm the RNAi knockdown was specifically targeting CSF-1-mediated proliferation, PKM were isolated, and cells from each fish (n=6) were split into two flasks. Immediately post-isolation, one flask from each fish was treated with CSF-1R specific dsRNA and all cultures were incubated for 18 hours. After incubation, cultures were left untreated or treated with either 10 ng/mL rgCSF-1 or 100 ng/mL rgGrn and monitored over 10 days for proliferation induced by each treatment both in CSF-1R knockdown cultures and in normal cultures.

4.2.10 The effects of in vivo administration of rgCSF-1

Analysis of the effects of rgCSF-1 on the *in vivo* circulating immune cell populations was analyzed in 4 fish *in vivo* using one experimental fish treated with 25 ng rgCSF-1, and one fish as a sham injected control. The reason for the small sample size was that the *in vivo* labelling with the BrdU reagent was extremely costly, in excess of $1,000/fish. The concentration of rgCSF-1used (10 ng/mL) was selected based on *in vitro* results that indicated that this concentration induced proliferation and differentiation responses in cultured/sorted PKMs. The analyses were done using cells from the peripheral blood and the kidney 24 hours post BrdU injection. Peripheral blood cells were isolated and the red cells were removed before flow cytometric analysis using the procedures described in chapter 3.

Kidney cells were cultured as described in chapter 3 for 6 days and on days 0, 2, 4 and 6 the cells were analyzed using flow cytometry, BrdU incorporation, and light microscopy. Because the accuracy of the *in* vivo BrdU labelling decreased after 6 days due to a depletion of the labelling agent, all the analysis was done up to that time point. The flow cytometric analysis was done as described in chapter 3. The cultures were observed employing the Nikon inverted microscope fitted with phase contrast 20X objective.

4.2.11 Generation of long-term kidney-derived cell cultures

Long term PKM cultures were derived from individual fish treated with 10 ng/mL of rgCSF-1. Recombinant goldfish CSF-1 suspended in incomplete medium was added to cultures on day 4 post- cultivation and was re-applied to each culture every 7 days. The status of each culture was monitored by counting the per cent of dead cells, as indicated by trypan blue exclusion, using a haemocytometer. Cultures were deemed to be non-sustainable when >80% of the cells stained positive with trypan blue.

4.2.12 Generation of long-term blood-derived cell cultures

Long term peripheral blood cell cultures were derived from individual fish and were treated with the same combinations and concentrations of growth factors as the long-term PKM cultures.

4.3 Results

4.3.1 Quantitative assessment of the CSF-1 mRNA in different tissues and sorted macrophage subpopulations

The expression of goldfish CSF-1 in tissues and in sorted progenitor cells, monocytes and macrophages was assessed using quantitative PCR. Goldfish CSF-1 mRNA levels were the highest in the spleen compared to the other tissues (Fig. 4.1A). CSF-1 mRNA levels in non-activated sorted goldfish monocytes was almost 4 times higher compared to that of progenitor cells or mature macrophages. Interestingly, this expression increased an additional 7 times compared to that observed in progenitor cells and mature macrophages after treatment of monocytes with phorbol esters (10 ng/mL of

PMA). PMA was found to significantly increase the expression of CSF-1 message in monocytes by almost 2 times, 12 hours after treatment, but had no significant effect on the CSF-1 expression in progenitor cells or mature macrophages (Fig. 4.1B).

4.3.2 The analysis of goldfish CSF-1 predicted peptide sequences

The predicted goldfish CSF-1 peptide was 199 amino acids and featured a secretion signal peptide cleaved at amino acid 29 (Fig. 4.2A). The peptide was approximately 22 kDa with a predicted isoelectric point of 6.6. Goldfish CSF-1 (Genbank accession number: EU045335) shared its highest amino acid identity with a putative zebrafish CSF-1 (XP001343870) at 89% and a putative trout CSF-1 (CAD88593) at 65%. The amino acid identity between goldfish and human CSF-1 (BAD92189) was only 27%. Importantly, cysteine residues responsible for the formation of the intra-chain and inter-chain disulphide bonds required for functional CSF-1 in mammals were present in the goldfish CSF-1 amino acid sequence. Although these cysteine residues were not in the same positions as the cysteines of the human CSF-1, the distance between the residues was almost identical between the goldfish and human sequences (Fig. 4.2A). RACE PCR identified three nucleotide sequence variants that differed in the 3' -untranslated region. The most commonvariant sequence was observed in 43 of 60 3'-RACE clones and for that reason this sequence was submitted to GenBank™. The full nucleotide sequence of allvariants contained TATA box, poly(A) signal, and poly(A)tail in the 3'-untranslated region, however the position and sequence of the Poly (A) signal differed between the variants (Fig. 4.2B).

4.3.3 *Goldfish rgCSF-1 binds to recombinant soluble CSF-1 receptor (sCSF-1R)*

One hundred µg of biotinylated goldfish rgCSF-1 and sCSF-1R were run on a SDS gel in conjunction with rgCSF-1 and sCSF-1R cross-linked using BS^3. The gel was transferred to nitrocellulose membrane and developed using HRP-conjugated avidin and enhanced chemiluminescent substrate ECL. The sCSF-1R M_r was approximately 31 kDa, and on SDS-PAGE it migrated as a doublet likely due to different glycosylation of the soluble receptor (Fig. 4.3A), and the M_r of the rgCSF-1 was approximately 26 kDa (Fig. 4.3B). In the cross-linked sample of rgCSF-1, two distinct bands were observed representing both monomeric rgCSF-1 as well as the homodimeric form with M_r of 50 kDa (Fig. 4.3C). In the sample containing cross-linked rgCSF-1 and sCSF-1R, multiple bands were observed; non-bound rgCSF-1 and sCSF-1R were observed at the predicted M_r, a band representing homodimeric rgCSF-1 was observed and also a higher M_r band at 90 kDa that represented sCSF-1R bound to the homodimeric form of rgCSF-1 (Fig. 4.3D). CSF-1 homodimerization and CSF-1-sCSF-1R binding was due to specific CSF-1-CSF-1 and CSF-1-sCSF-1R interactions as no cross-linking was observed in the BSA peptide cross-linking control sample (Fig. 4.3E).

4.3.4 *Goldfish rgCSF-1 induced differentiation of goldfish monocytes into macrophages*

Recombinant goldfish CSF-1 was added to sorted goldfish progenitor cells, monocytes and macrophages 2 days post-cultivation at concentrations of 50 ng/mL, 25 ng/mL, 10 ng/mL, 5 ng/mL or 1 ng/mL. Cell growth was monitored daily using flow cytometry and the rgCSF-1 treated cell cultures were compared to those treated with CCM (positive control), elution buffer, and non-treated cultures. Progenitor cells and macrophages were not affected by the addition of rgCSF-1. However, sorted monocytes were induced to differentiate into mature macrophages much earlier than those incubated in the presence of CCM. By day 4 post-treatment 62% of the 25000 total events were recorded in the macrophage gate in the rgCSF-1 treated cells compared to 3% in the CCM control. On day 8 post treatment the rgCSF-1 treatment had 47% macrophages compared to 29% for the CCM control. The rgCSF-1-induced transition of monocytes to macrophages was observed as early as day 2 post treatments, which was more than 3 days earlier than cultures treated with CCM (Fig 4.4).

4.3.5 Goldfish rgCSF-1 induced proliferation of sorted goldfish primary kidney macrophage subpopulations

Different amounts of rgCSF-1 were used to assess the capacity of the growth factor to induce proliferation of FACS-sorted goldfish progenitor cells, monocyte and macrophages (Fig 4.5). Recombinant CSF-1 induced proliferation in sorted monocytes and macrophages and, to a lesser extent, progenitor cells. The proliferative response was dose dependant where 1 ng/mL rgCSF-1 induced the lowest response, whereas cells treated with 10 ng/mL to 50 ng/mL of rgCSF-1 exhibited very strong proliferative response. The proliferative response observed in the monocyte cultures was normalized by subtracting the values for non-treated cell cultures. As shown in Fig. 4.5B, 10 ng/mL of rgCSF-1 induced a significantly higher proliferative response compared to that induced by CCM (positive control), as determined by one way ANOVA ($P < 0.05$). In contrast, 10 ng/mL of rgCSF-1 induced consistently higher, but not significant, proliferation of mature macrophages from day 2 of cultivation onwards (Fig. 4.5C). Although CCM induced a stronger proliferative response in progenitor cells when compared to any of the rgCSF-1 treatment groups, the addition of 10 ng/mL, 25 ng/mL or 50 ng/mL of rgCSF-1 did enhance the proliferation of progenitor cells four days after addition of rgCSF-1 (Fig. 4.5A). CSF-1 elution buffer (negative control) did not induce or inhibit the proliferative response in any of the experiments.

Pre-incubation of sCSF-1R with the rgCSF-1 at a ratio of 50 ng/mL of sCSF-1R to 10 ng/mL of rgCSF-1, abrogated the proliferative response observed in the sorted monocyte and macrophage populations compared to those treated with rgCSF-1 alone (Fig. 4.5 A, B, C). Addition of sCSF-1R to the cultures treated with CCM also inhibited cell proliferation, but not to the same extent when compared to that observed for the rgCSF-1 treated cultures. Treatment of sorted progenitor cells, monocytes and macrophages with 50 ng/mL of sCSF-1R alone, had no significant effect on cell proliferation (Fig. 4.5 A, B, C).

The proliferative response induced by rgCSF-1 in sorted monocytes or macrophages was also inhibited by pre-incubation of sorted cell subpopulations with the affinity purified rabbit anti-CSF-1R IgG that recognized the first two extracellular Ig binding domains of the CSF-1R [49]. The addition of different amounts of the anti-CSF-1R IgG to sorted progenitor cells (Fig. 4.6A), monocytes (Fig. 4.6B) or macrophages (Fig. 4.6C) for 2 hours at room temperature before addition of the ligand (rgCSF-1) resulted in a dose-dependent inhibition of cellular proliferation. Maximal inhibition was observed after addition of 100 ng/mL of anti-CSF-1R. Addition of 100 ng/mL of anti-

CSF-1R by itself to the cell cultures increased expression of TNF-α and IL-1β but had no effect on cellular proliferation when compared to that of non-treated cell cultures (Fig. 4.6 A, B, C).

4.3.6 Detection of reactive oxygen and nitrogen intermediates after treatment of primary kidney macrophages with rgCSF-1 alone or in combination with rTNF-α

Recombinant goldfish CSF-1 induced production of the reactive oxygen intermediates but not reactive nitrogen intermediates. Nitric oxide production was measured at two time points, 48 hours and 72 hours post-treatment. Different amounts of rgCSF-1 were added to cultures containing 5 x 10^5 cells each, and the resulting nitrite values for each cultures did not exceeded 5 μM, and were found not to be significantly different from those of medium controls. In contrast, treatments of PKM with 100 ng/mL of rTNF-α (positive control) induced a significant nitric oxide response where 14 μM and 23 μM nitrite were present in cultures at 48 and 72 hours post-treatment, respectively. Treatment of PKM with both 100 ng/mL of rTNF-α and 10 ng/mL of rgCSF-1 induced a significant nitric oxide response in PKM; 15 μM and 22 μM nitrite at 48 and 72 hours post treatment, respectively. Results obtained from the combined treatment suggest that there are no additive or synergistic effects for the induction of nitric oxide production in goldfish macrophages after stimulation with rTNF-α and rgCSF-1 (Fig. 4.7A).

Production of reactive oxygen intermediates was up regulated by rgCSF-1. (Fig. 4.7B). rgCSF-1 induced respiratory burst activity at concentrations of 1 ng/mL, 5 ng/mL and 10 ng/mL. rgCSF-1 initiated an early respiratory burst response when PKM were treated with 5 ng/mL and 10 ng/mL, where higher respiratory burst activity was observed at 5 hours compared to that observed at 20 hours post-treatment (Fig. 4.7 B). When rgCSF-1 and rTNF-α were added to cell cultures in combination, a strong respiratory burst response was observed at 5 hours post-treatment which was reduced 20 hours post treatment. Combined treatment with rgCSF-1 and rTNF-α did not result in an additive or synergistic induction of the production of reactive oxygen intermediates by goldfish macrophages. Treatment of goldfish macrophage with PMA alone (priming control) did not induce the production of reactive oxygen intermediates (Fig. 4.7B).

4.3.7 Recombinant goldfish rgCSF-1 induced chemotaxis in goldfish macrophages

Goldfish rgCSF-1 induced a dose-dependent chemotactic response of goldfish macrophages. Cell migration was measured by counting 20 random fields of view from the underside of the chemotaxis filter under the 100X objective for each of the sets of treatments (n=4). Medium alone controls had an average of 6.5 cells per field of view, whereas the rTNF-α positive control had an average of 15 cells per field of view. rgCSF-1 treatments of 1 ng/mL, 5 ng/mL, or 10 ng/mL resulted in enhanced chemotactic response where averages of 10, 15.5 and 20 cells per field of view were recorded respectively. When rgCSF-1 and rTNF-α were added in combination at concentrations of 10 ng/mL and 100 ng/mL, respectively, an average of 13 cells per field of view were observed. Chemokinesis controls for rgCSF-1 and rgCSF-1 + rTNF-α treatment confirmed that the chemotactic response was directional and was not due to chemokinesis (Fig 4.8)

4.3.8 Goldfish rgCSF-1 enhanced phagocytosis in goldfish macrophages

Treatment of goldfish macrophages with rgCSF-1 enhanced their ability to engulf synthetic beads. Phagocytosis was measures using flow cytometry-based assay where cells containing 3 or more fluorescent beads were considered to be highly phagocytic. While only 5% of the medium alone treated cells (controls) phagocytosed 3 or more beads, 15% and 12% of PKM treated with 100 ng/mL rTNF-α or MAF (positive controls) phagocytosed 3 or more beads, respectively (Fig. 4.9). Treatment of macrophages with 1 ng/mL, 5 ng/mL or 10 ng/mL of rgCSF-1 increased the phagocytic activity of PKM, such that 15%, 18% and 16.5% of cells were observed to have engulfed 3 or more beads, respectively. The phagocytic response appeared to be the highest after treatment of PKM with 5 ng/mL of rgCSF-1, but was not statistically different from that induced by other doses of rgCSF-1 ($P < 0.05$, one-way ANOVA). The phagocytic response of rgCSF-1-treated macrophages was significantly higher than that of medium control group ($P < 0.05$, one-way ANOVA). The addition of 100 ng/mL of rTNF-α to any of the rgCSF-1-treated PKM cultures resulted in comparable percent phagocytosis to that induced by rgCSF-1-alone. Combined treatment of PKM with the two cytokines resulted in 14%, 15.5% and 17% of macrophages that engulfed 3 or more beads induced by 1 ng/mL, 5 ng/mL or 10 ng/mL of rgCSF-1 with 100 ng/mL of rTNF-α, respectively (Fig. 4.10).

4.3.9 *RNAi knockdown of CSF-1 receptor in goldfish primary kidney macrophages abrogated proliferation and differentiation induced by recombinant CSF-1*

RNAi knockdown of CSF-1R was performed to confirm the specificity of goldfish rgCSF-1 for the CSF-1R. Experiments performed previously in which 10 ng/mL of rgCSF-1 induced a significant proliferative response in sorted monocytes and their differentiation into macrophages (Fig. 4.4 and 4.5), were repeated using cells in which the CSF-1R had been knocked down. RNAi induced knockdown of CSF-1R was confirmed using non-isotopic Northern blot and RT-PCR analysis comparing CSF-1R expression to β-actin (Fig. 4.11). CSF-1R expression was observed to be knocked down significantly using 5 nM or higher (7.5 nM, 10 nM or 15 nM) of CSF-1R-specific oligos, as indicated by Northern blot analysis, and with 10 nM or more (15 nM) of CSF-1R-specific oligos, using RT-PCR. After knockdown was confirmed, proliferation and flow cytometry differentiation assays were performed using the same treatments as before (see section 4.2.4 and 4.2.5); to assess rgCSF-1 induced proliferation and differentiation of sorted RNAi treated monocytes.

CSF-1-induced proliferation was significantly abrogated after knockdown of CSF-1R using 10 nM of CSF-1R-specific oligos (Fig. 4.12). Treatment of sorted goldfish monocytes with 10 ng/mL of rgCSF-1 induced a characteristic proliferative response in cells not treated with RNAi oligos, in cells treated with 293fectin transfection reagent, in cells treated with oligos without 293fectin, and non-treated cells. Monocytes treated with CSF-1R-spcific oligos pre-incubated with 293fectin exhibited a significant inhibition of proliferation after rgCSF-1 treatment ($P < 0.05$, one-way ANOVA). The difference in mean number of cells per mL between the RNAi-treated cells stimulated with rgCSF-1 and cells grown in medium alone was significant ($P < 0.05$, one-way ANOVA). Control PKM cultures incubated with rgCSF-1R-specific RNAi oligos + 293fectin and then stimulated using recombinant goldfish granulin, exhibited a proliferative response similar in intensity and duration to cells treated with granulin and not incubated with RNAi oligos (Fig. 4.13).

Differentiation of monocytes into macrophages induced by rgCSF-1 was blocked by knockdown of the CSF-1R in sorted goldfish monocytes (Fig. 4.14). Treatment of monocytes with 10 nM of CSF-1R-specific RNAi oligos prior to treatment with 10 ng/mL of rgCSF-1 abrogated the transition of monocytes into macrophages (Fig. 4.14). Monocytes treated with the 293fectin transfection reagent alone or rgCSF-1R-specific oligos alone caused no change in the observed rgCSF-1 induced differentiation.

4.3.10 Detection of native CSF-1 in goldfish primary kidney macrophage culture supernatants

Using the sCSF-1R as a probe on a Far Western blot, native CSF-1 was detected in 10X concentrated goldfish PKM culture supernatants (Fig 4.15). Albumin was removed from CCM before concentration and, once concentrated, the CCM was run on SDS-PAGE and transferred to nitrocellulose for Western blot analysis. The CCM lanes were then incubated with 10 ng/mL, 25 ng/mL, 50 ng/mL, or 100 ng/mL of recombinant sCSF-1R as the primary agent. All concentrations of sCSF-1R were able to bind to a sufficient amount of native CSF-1 to be detected once the blot was developed (Fig. 4.15).

4.3.11 *Supernatants from CCL-71 cell cultures induced goldfish primary kidney macrophage proliferation that was abrogated by soluble CSF-1 receptor*

The presence of native CSF-1 in the supernatants of CCL-71 cultures suggested that CCL-71 supernatants may be used as a substitute for PKM CCM normally employed to establish goldfish PKM *in vitro*. I performed a BrdU analysis and compared the proliferation of cells in cultures to which the following was added: CCL-71 supernatant ± 50 ng/mL of sCSF-1R, PKM CCM ± 50 ng/mL of sCSF-1R, 10 ng/mL of rgCSF-1 ± sCSF-1R or incomplete medium ± 50 ng/mL of sCSF-1R. Cells treated with CCL-71 supernatants, PKM CCM (CCM), rgCSF-1 or CCL-71 supernatant exhibited significant proliferative response (Fig. 4.16). The addition of the sCSF-1R inhibited proliferation of goldfish macrophages in all treatment groups (Fig. 4.16). Interestingly, addition of the sCSF-1R had a more significant impact on CCL-71 supernatant- induced proliferation compared to that induced by PKM CCM (Fig. 4.16), suggesting that the proliferative response induced by CCL-71 supernatants may be primarily due to CSF-1. Although there were no significant differences in the proliferative response induced by either rgCSF-1, PKM CCM or CCL-71 supernatants, there were significant differences in the proliferative responses induced by each of the treatments when compared to those of medium controls ($P < 0.05$, ANOVA) (Fig. 4.16).

4.3.12 *Injection of rgCSF-1 induced an increase in circulating monocytes in vivo*

In vivo addition of approximately 10 ng of rgCSF-1/mL of blood to goldfish caused a significant increase in circulating monocytes two days post injection (Fig. 4.17). Circulating monocytes were measured by analyzing the size and internal complexity of isolated blood mononuclear cells in addition to analyzing the fluorescence emitted by cells that had undergone DNA synthesis and incorporated the BrdU reagent. The monocyte population was observed to increase from approximately 34% of isolated cells to approximately 47% of isolated cells. Of the monocytes observed in sham controls, less than 1% were labelled by BrdU, whereas approximately 13% of the cells were labelled in the fish injected with rgCSF-1 (Fig. 4.17). Interestingly, all treatments including sham injected controls had a highly labelled population of cells of low internal complexity and small size. These cells likely represent lymphocytes and thrombocytes that have been reported to be present in the pool of mononuclear blood cells of both carp and goldfish [41].

Goldfish cells isolated from the kidney were cultured at 2 days post-injection and analyzed on days 0, 2, 4 and 6 post-cultivation. Flow cytometric data were analyzed by dividing the number of BrdU labelled cells in a particular gate by the total number of cells in the gate, thereby providing the per cent of cells in each gate that proliferated post-BrdU labelling. Treatment with rgCSF-1 induced an increase of monocytes and macrophages in cultures compared to sham injected control (Fig. 4.18). By day 2 of cultivation cells labelled with BrdU were observed for all treatment groups, although higher number of labelled cells were observed in cultures established from rgCSF-1-injected fish. By day 6 of cultivation, BrdU labelled cells from the rgCSF-1 treated fish were present in all cytometer gates where the majority (72% of total number of fluorescing cells) were monocytes. Compared to the sham injected control, cell cultures established from the rgCSF-1-injected goldfish had 28% more fluorescing monocytes and 5% more fluorescing macrophage on day 4, and 3% more fluorescing monocytes and 39% more fluorescing macrophages on day 6 post isolation (Fig. 4.18). Due to the fact that these *in vivo* studies were done using a single fish, the results were not analyzed for statistical significance. Rather, the per cent changes in the BrdU-labelled cells cultures should be used as preliminary indicators of biological trends induced by injection of rgCSF-1.

4.3.13 Analysis of long-term kidney and blood-derived cultures

The long-term cultures from goldfish kidney leukocytes were maintained for up to 160 days by weekly addition 10 ng/mL of rgCFS-1 in ‘proof of principal’ experiments (Fig. 4.19). Morphologically, the primary macrophage cultures were similar to those maintained by the addition of CCM. Furthermore, the progression of cell development and proliferation was also similar to that induced by CCM, where by day 6 post-cultivation three distinct subpopulations of cells were evident as analyzed by flow cytometry; progenitor cells, monocytes and mature macrophages. By day 21 of cultivation (i.e. following 3 rgCSF-1 additions) cultures of primarily adherent cells were observed, compared to the non-treated control cultures where almost all of the cells had died (Fig. 4.20). The rgCSF-1 treated cultures were demonstrated to be primarily macrophages by flow cytometry, and when activated with MAF and LPS, they produced nitric oxide, indicating that they were functionally mature macrophages (Fig 4.21). The mean ± SEM doubling time of rgCSF-1-maintained cultures was 96 hours ± 7.5 hours.

Long term cultures were also established using peripheral blood leukocytes. Weekly supplementation of these cultures with 10 ng/mL of rgCSF-1 resulted in cultures primarily composed of monocytes and macrophages by day 49 post isolation (7 rgCSF-1 additions). The cultures were a mix of adherent cells and suspended cells until day 63 of cultivation, after which the monocytes disappeared and only macrophages remained in the cultures until the end of the observation period (120 days). Upon activation by MAF and LPS, cells obtained from these cultures produced a strong nitric oxide response, suggesting that they were indeed mature macrophages. The mean doubling time for cell cultures established from peripheral blood leukocytes and supplemented with rgCSF-1 was 102 hours ± 12 hours.

4.4 Discussion

In this chapter, I described the identification and functional characterization of goldfish CSF-1 molecule, which is the first comprehensive analysis of this molecule in lower vertebrates. Goldfish CSF-1 has 199 amino acids and was significantly smaller than the mammalian CSF-1 isoforms (secreted glycoprotein or the secreted/matrix bound proteoglycan form of mammalian CSF-1) and similar to the membrane bound glycosylated form of the molecule [428]. Interestingly, all mammalian CSF-1 isoforms were shown to be functional where the N-terminus 150 amino acids were required for proper folding and function [426]. Goldfish CSF-1 was most similar to zebrafish and trout CSF-1 molecules which have been recently identified but not functionally characterized. It shared the highest sequence identity and structure with zebrafish CSF-1 and had an identical cysteine spacing pattern.

Goldfish CSF-1 was highly expressed in the spleen, suggesting that the spleen may be a primary site of the macrophage/monocyte development. Although the kidney appears to be the primary location of hematopoiesis in bony fishes [24, 40, 41, 43], it is possible that non-differentiated cells enter the circulation and are directed down the final steps of the myeloid pathway in the spleen or by the CSF-1 synthesized in the spleen.

When I examined the CSF-1 expression in sorted goldfish progenitor cells, monocytes and macrophage, I found it to be highly expressed in the monocyte subpopulation. It has been demonstrated that fish macrophages produce their own autocrine/paracrine growth factors [24, 46, 47]. The secretion of endogenous growth factors has been primarily attributed to the mature macrophage subpopulation of the PKM [44]. Thus, although the monocyte population may express the highest levels of CSF-1 message this may not directly correlate to a higher translation of active CSF-1 protein by the monocytes. Furthermore, mammalian CSF-1 has been shown to be synthesized by monocytes following activation with LPS or PMA, but not without this stimulation [414].

The treatment of sorted goldfish progenitor cells, monocytes and macrophages and subsequent treatment of these cells with PMA resulted in an increase in CSF-1 expression in the monocyte subpopulation. Assessment of the protein expression of CSF-1 in sorted populations of progenitor cells, monocytes and macrophages left untreated or treated with LPS or PMA appear to mirror the mammalian regulation of CSF-1.

Monocytes stimulated with LPS or PMA produce CSF-1 protein, whereas those that were not treated did not produce CSF-1 despite the expression of high mRNA levels by this subpopulation of PKM.

The induction of proliferation by goldfish rgCSF-1 was blocked by treatment with an affinity-purified rabbit IgG antibody generated against the first two immunoglobulin binding domains of the CSF-1R which have been shown to be required for CSF-1 function in mammals [133]. Addition of anti-CSF-1R antibody slightly increased of the mRNA expression of goldfish TNF-α and IL-1β, which are pro-inflammatory cytokines. This may be due to cross-linking events occurring when the antibodies bind to receptors on the surface of the cells [444, 445]. Importantly, this increase in pro-inflammatory gene expression did not coincide with a change in the base levels of proliferation seen in non-treated cells.

Further demonstration of the specificity of the rgCSF-1 was demonstrated by RNAi knockdown of the goldfish CSF-1R, which abrogated rgCSF-1-induced monocyte proliferation and their differentiation into macrophages. Interestingly, the proliferative effect induced by rgCSF-1 was also abrogated by addition of a recombinant goldfish sCSF-1R. We have shown previously that the addition of sCSF-1R to actively growing goldfish macrophage cultures significantly inhibited their proliferation *in vitro* as measured by BrdU incorporation [49]. The ability of sCSF-1R to dose-dependently inhibit proliferation induced by rgCSF-1, as well as the ability of rgCSF-1 to bind to sCSF-1R, suggests that this may be a novel mechanism of self-regulation of macrophage development in bony fish. Whereby the sCSF-1R is alternatively produced in place of cell surface CSF-1R in order to facilitate the functional removal of circulating CSF-1 from specific areas where monocyte/macrophage proliferation or differentiation are no longer necessary or potentially damaging.

Although the work I have done in the goldfish has focused largely on the functional properties of CSF-1, work done in the rainbow trout, zebrafish and goldfish has shed light on the genetic aspects of teleost CSF-1 regulation and possible isoforms produced as functional proteins [243]. In this study, two paralogous CSF-1 genes were identified in the zebrafish that reside on CH11 and CH9. Syntenic analysis showed the relationship of these two paralogous chromosomal regions to the region on human CH1 which harbours the human CSF-1 gene. This suggests that the two zebrafish CSF-1 paralogs have arisen from either a chromosome or whole genome duplication event, coinciding with the whole genome duplication known to have occurred in the teleost fish [446]. Also identified in this study were three fish CSF-1 genes that had a unique exon/intron structure (7, 9 or 10 exons) compared to 9 exons in mammals, with exception of primates that have 10 exons. Interestingly, because it contains 10 exons and 9 introns, the trout CSF-1 gene might represent the ancestral CSF-1 gene, and zebrafish CSF-1(2) and mammalian CSF-1 genes which have all lost intron 6, as well as the zebrafish CSF-1(1) that lost all the 3 introns at the 3'-end likely represent the more derived form of CSF-1 [243].

When I analyzed the potential splice variants of teleost CSF-1, it appears that bony fish do not possess the same splice variants reported for mammals. By alternative splicing of exon 6, and alternative use of one of the two exons for the 3'-UTR, the mammalian CSF-1 gene can produce multiple transcripts with three different translations [149, 151, 165]. No significant splicing variants were detectable upon analysis of zebrafish or trout CSF-1 genes [243]. Thus, the mammalian equivalent alternative splicing variants were not present in trout and are likely not present in zebrafish or the goldfish. These observations suggest interesting differences exist between teleost CSF-1 and mammalian CSF-1. Although the exact post-translational modifications of teleost CSF-1 are still unknown, it has been shown that of the three splice variants seen in mammals, the secreted proteoglycan form is the most abundantly produced in fish [165]. Moreover, the presence of both a membrane-bound, as well as two soluble forms of mammalian CSF-1, allow for a range of target cell types and functional diversity that may be influenced by different microenvironment [133]. The possibility that teleosts lack some of these splice variants suggests that perhaps some of the functions attributed to CSF-1 may be specific to mammals only. Interestingly, CSF-1 has been implicated in bone remodelling by affecting the growth of osteoclasts in the bone marrow [177, 447-449], and CSF-1 has also been shown to play a critical role in foetal development, pre-implantation, survival and placental development [192, 450-453]. These functions are absent in teleost CSF-1 because teleost fish do not possess bone marrow and are oviparous and therefore do not require maternal CSF-1 production for proper embryo development or the generation of a suitable microenvironments for development. It will be interesting to determine whether teleost CSF-1 has a role in bone formation and/or development.

Despite being commonly associated with developmental processes, CSF-1 has a diversity of immunostimulatory effects that rival any cytokine. Mammalian CSF-1 has been shown to enhance cytotoxicity, superoxide production, phagocytosis, chemotaxis and cytokine production in monocytes and macrophages [438, 454]. CSF-1 can prime some innate immune responses while suppressing others by modulation of Toll-like receptors (TLRs). For example, priming of monocytes and macrophages with CSF-1 down-regulated expression of TLR1, TLR2, TLR6 and TLR9, and has no effect on TLR4 [455], but increased the expression of CD14, the LPS receptor cluster [456]. Thus, CSF-1 enhanced cytokine production in response to LPS, but suppressed the CpG DNA response [455]. CSF-1 up regulated human monocyte expression of the P2X7 extracellular ATP receptor [457], that controls dendritic cells (DC) and macrophage inflammatory functions, including intracellular bacterial killing and favours the generation of cytokines that stimulate T-helper 2 responses [458]. In addition to modulating immunological processes itself, CSF-1 has been shown to regulate the expression of a number of cytokine which have their distinct effects on immune responses. CSF-1 has been shown to increase the expression of different Fc receptors Fcγ III, Fcε II and Fcγ I, which were up regulated after exposure of monocytes to CSF-1 [456]. Fc receptor changes are commonly accompanied by increases in the expression of pro-inflammatory and chemotaxis-inducing cytokines, such as IL-8, IL-6, IL-18 and TNF-α, as well as HLA-I and HLA-II which are involved in antigen presentation [3, 456].

Functionally, goldfish and mammalian CSF-1s appear to be similar in regards to the proliferation of monocytes and macrophages. After addition of CSF-1 to the fish, circulating monocyte numbers were found to increase significantly. Moreover, the increased population of monocytes seen in the blood were cells that had been recently developed as indicated by the BrdU incorporation into their DNA. These results are similar to those obtained when mice were injected with CSF-1 where the *in vivo* circulating monocyte pool also increased [459].

Enhanced cytotoxicity, superoxide production, phagocytosis, chemotaxis and cytokine production have been shown in this study to be processes modulated by goldfish CSF-1 and this has also been shown to be the case for mammalian CSF-1 [166]. Interestingly, mammalian CSF-1 has been shown to act in combination with mammalian TNF-α to enhance cellular response and a number of biological processes including the induction of proliferation [441, 442, 460], respiratory burst activity [166], and osteolysis [448]. However, this does not appear to be the case for goldfish rgCSF-1, which I have shown above not to have additive or synergistic activity with goldfish rTNF-α.

Goldfish CSF-1 represents the first CSF-1 to be functionally characterized in fish and is also the first colony stimulating factor functionally characterized for any non-mammalian organism. Although goldfish CSF-1 was significantly smaller than mammalian CSF-1, it featured the required amino acids in the right positions to form a functional peptide. Goldfish CSF-1 was functionally similar to the mammalian CSF-1 since it induced the differentiation and proliferation of fish mononuclear cells by binding to the CSF-1R. Interestingly, goldfish fibroblast cell line (CCL-71) expressed and secreted copious amount of CSF-1, which was similar to that reported for mammalian L-929 cells [433]. That CCL-71 cells produced CSF-1 which induced proliferation of goldfish macrophages was confirmed in studies where the addition of the sCSF-1R abrogated this proliferative response in a dose-dependent manner.

Establishment of long-term fish macrophage cell cultures and a few cell lines has long been possible without transformation of the cells [461]. Historically these cultures have been induced to grow by stimulation with phorbol esters and calcium ionophore or even simply in the presence of foetal bovine serum [462-464]. Interestingly, fish cell lines and long term cell cultures have been generated initially by supplementation with cell-conditioned medium produced by a feeder cell population [465, 466]. Although this phenomenon is commonly observed in mammalian cell cultures [433], specific exogenous growth factors have never been used to generate non-transformed spontaneously growing cell lines. Due to the unique nature of fish cells, there is a possibility that specific growth factors that have been functionally linked to proliferative processes in fish may be useful for the establishment of long-term fish cell lines.

Addition of recombinant CSF-1 to goldfish PKM cultures, which normally grow for a maximum of 2 weeks before entering a period of senescence and apoptosis [24, 44, 53], resulted in an extension of the life-span of the cultures for up to 160 days. Interestingly, CSF-1 significantly extended the life span of macrophage cultures when compared to those treated with CCM or untreated controls. Addition of rgCSF-1 resulted in both PKM and peripheral blood mononuclear cell (PBM) cultures to develop primarily into adherent macrophage cultures that were successfully maintained by weekly addition of rgCSF-1 for up to 160 days. The cells from these cultures produced nitric oxide, which is one of the indicators of their differentiation into being mature fully functional macrophages [418, 467, 468].

Although goldfish CSF-1 appears to act in a very similar manner to mammalian CSF-1, it should be noted that its effects may not be regulated by the same mechanisms. Mammalian CSF-1 was shown to be rapidly removed from the circulation by sinusoidal macrophages, primarily by Kupffer's cells in the liver. In mammals, CSF-1 has been shown to be recognized by the membrane-bound CSF-1R and the entire complex internalized and destroyed by macrophages. Thus, CSF-1 levels were shown to be regulated by the number of macrophages in the sinuses [225]. It is unclear whether this internalization process is the clearance mechanisms for CSF-1 in fish. However, our laboratory has identified a soluble CSF-1 receptor in the goldfish [49] and shown that it efficiently bound CSF-1 thereby abrogating the proliferative effect mediated by the ligand. Thus may be a novel, teleost specific, clearance mechanisms for CSF-1, by which fish macrophages may control their own development.

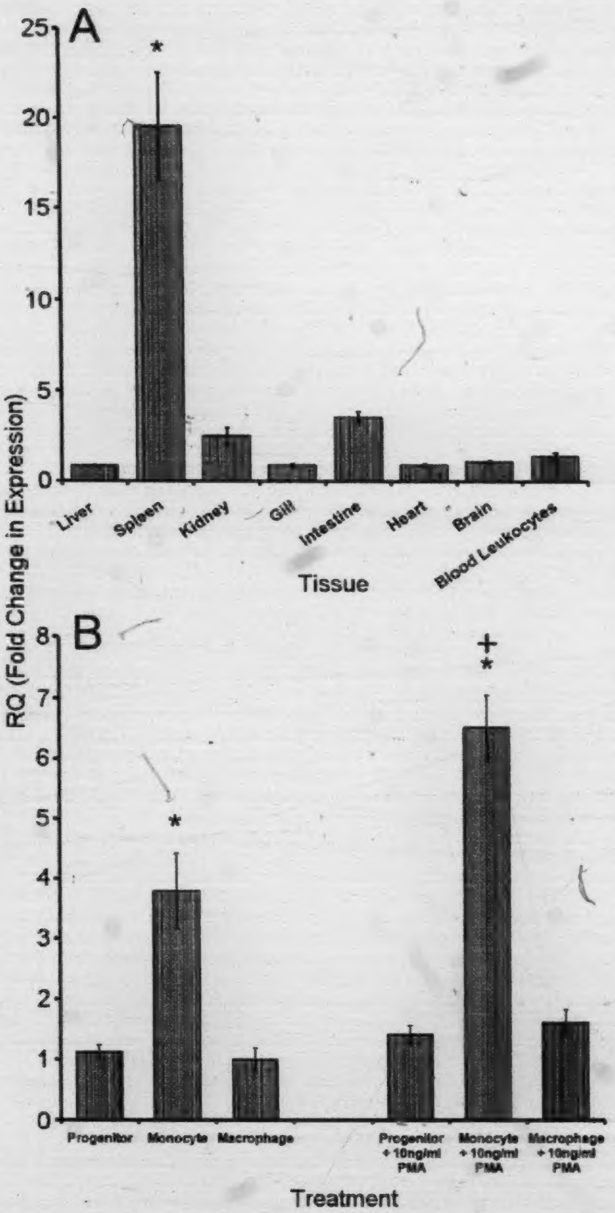
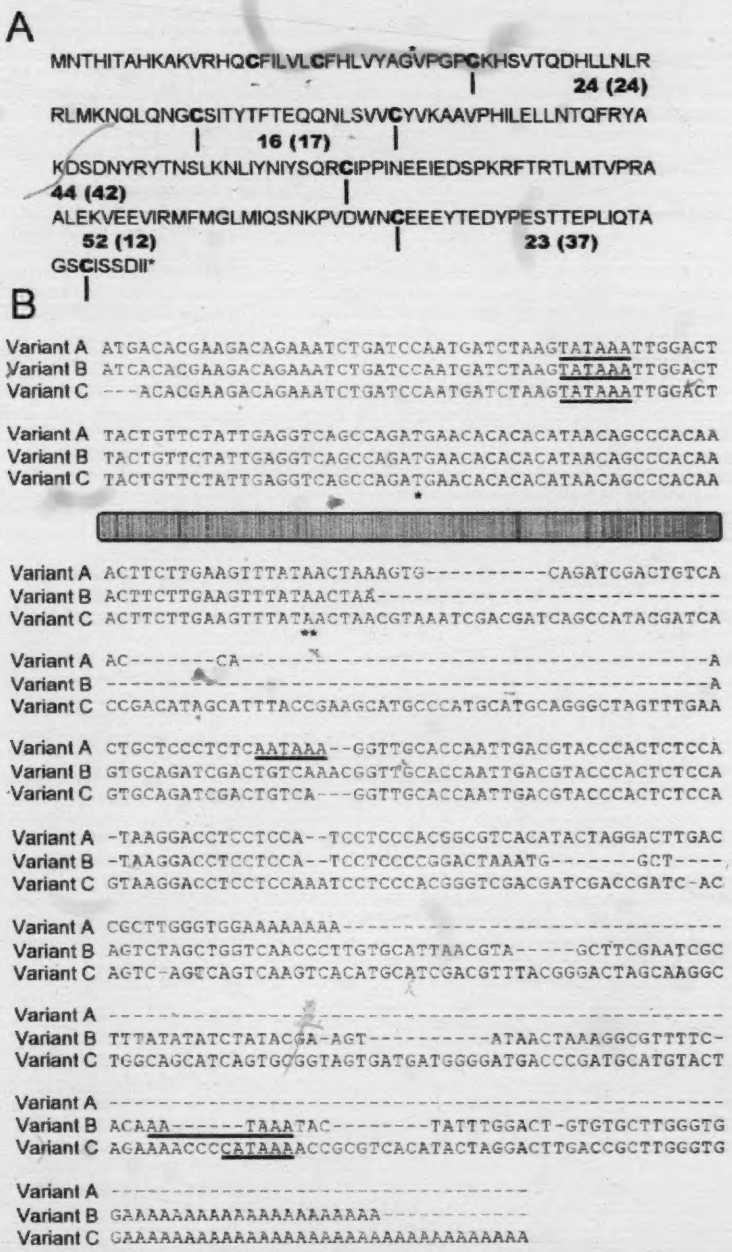


Figure 4.1 Quantitative PCR analysis of goldfish CSF-1 mRNA expression.(A) expression in different tissues. (B) The expression in sorted progenitor cells, monocytes and macrophages non-treated and treated with 10ng/mL PMA. Both PCRs were run in parallel with the endogenous control β-actin, and normalized against results for the liver (A) or sorted macrophages (B). The expression analysis was done using tissues and primary kidney macrophages isolated from individual fish (n=6). Statistical significancewas determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*) for comparison to the normalized sample and (+) denotes significance between PMA treated and non-treated monocytes. Each bar represents mean ± SEM (n=6).



A

MNTHITAHKAKVRHQ**C**FILVL**C**FHLVYAGVPGP**C**KHSVTQDHLLNLR
24 (24)
RLMKNQLQNG**C**SITYTFTEQQNLSVV**C**YVKAAVPHILELLNTQFRYA
16 (17)
KDSDNYRYTNSLKNLIYNIYSQR**C**IPPINEEIEDSPKRFTRTLMTVPRA
44 (42)
ALEKVEEVIRMFMGLMIQSNKPVDWN**C**EEEYTEDYPESTTEPLIQTA
52 (12)
23 (37)
GS**C**ISSDII*

B

```
Variant A ATGACACGAAGACAGAAATCTGATCCAATGATCTAAGTATAAATTGGACT
Variant B ATCACACGAAGACAGAAATCTGATCCAATGATCTAAGTATAAATTGGACT
Variant C ---ACACGAAGACAGAAATCTGATCCAATGATCTAAGTATAAATTGGACT

Variant A TACTGTTCTATTGAGGTCAGCCAGATGAACACACACATAACAGCCCACAA
Variant B TACTGTTCTATTGAGGTCAGCCAGATGAACACACACATAACAGCCCACAA
Variant C TACTGTTCTATTGAGGTCAGCCAGATGAACACACACATAACAGCCCACAA
                                   *

Variant A ACTTCTTGAAGTTTATAACTAAAGTG----------CAGATCGACTGTCA
Variant B ACTTCTTGAAGTTTATAACTAA----------------------------
Variant C ACTTCTTGAAGTTTATAACTAACGTAAATCGACGATCAGCCATACGATCA
                            **

Variant A AC-------CA--------------------------------------A
Variant B -------------------------------------------------A
Variant C CCGACATAGCATTTACCGAAGCATGCCCATGCATGCAGGGCTAGTTTGAA

Variant A CTGCTCCCTCTCAATAAA--GGTTGCACCAATTGACGTACCCACTCTCCA
Variant B GTGCAGATCGACTGTCAAACGGTTGCACCAATTGACGTACCCACTCTCCA
Variant C GTGCAGATCGACTGTCA---GGTTGCACCAATTGACGTACCCACTCTCCA

Variant A -TAAGGACCTCCTCCA--TCCTCCCACGGCGTCACATACTAGGACTTGAC
Variant B -TAAGGACCTCCTCCA--TCCTCCCCGGACTAAATG-------GCT----
Variant C GTAAGGACCTCCTCCAAATCCTCCCACGGGTCGACGATCGACCGATC-AC

Variant A CGCTTGGGTGGAAAAAAAA-------------------------------
Variant B AGTCTAGCTGGTCAACCCTTGTGCATTAACGTA-----GCTTCGAATCGC
Variant C AGTC-AGTCAGTCAAGTCACATGCATCGACGTTTACGGGACTAGCAAGGC

Variant A --------------------------------------------------
Variant B TTTATATATCTATACGA-AGT----------ATAACTAAAGGCGTTTTC-
Variant C TGGCAGCATCAGTGCGGTAGTGATGATGGGGATGACCCGATGCATGTACT

Variant A --------------------------------------------------
Variant B ACAAA------TAAATAC--------TATTTGGACT-GTGTGCTTGGGTG
Variant C AGAAAACCCCATAAAACCGCGTCACATACTAGGACTTGACCGCTTGGGTG

Variant A ----------------------------------
Variant B GAAAAAAAAAAAAAAAAAAAAA------------
Variant C GAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 4.2 The predicted goldfish CSF-1 amino acid sequence. (A) Cysteine residues are bolded and the secretion signal peptide predicted cleavage site is marked (*). Bolded lines indicate cysteine residues that are important for folding and homodimerization in mammalian CSF-1. Numbers indicate the distance between each cysteine residue in the goldfish sequence and in brackets the distance between the corresponding cysteine in human CSF-1. (B) The nucleotide sequence showing TATA box, polyA signal (underlined), the start codon (*) and stop codon (**) of the three goldfish CSF-1 variants.

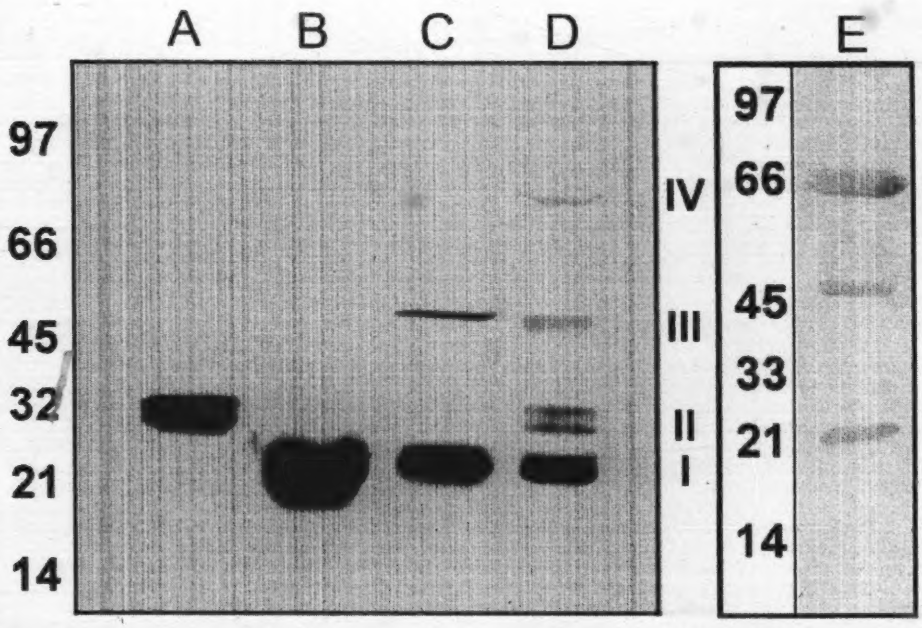


Figure 4.3 Western blot showing homodimer formation of rgCSF-1 and binding of rgCSF-1 to a recombinant soluble CSF-1R.(A) the recombinant soluble goldfish CSF-1R alone (II); (B) recombinant goldfish CSF-1 (I); (C) recombinant goldfish CSF-1 cross-linked with bis (sulfosuccinimidyl) suberate BS^3(III); (D) recombinant goldfish CSF-1 and recombinant goldfish soluble CSF-1R cross-linked with BS^3(IV). rgCSF-1 (50kDa (homodimeric form), and 24 kDa (non-cross-linked form)) and 100 µg BSA (66 kDa) cross-linked with BS^3 served as a cross-linker control, demonstrating that non-interacting peptides did not cross-link when BS^3 was added (E)

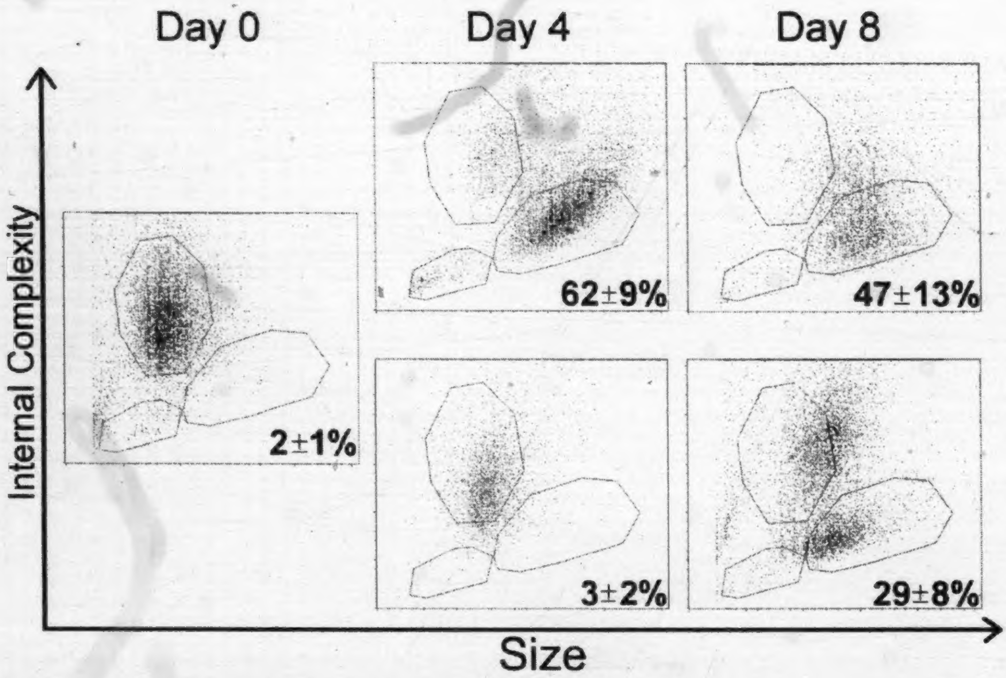


Figure 4.4 rgCSF-1 induces monocyte to macrophage differentiation. Flow cytometric analysis showing the differentiation of goldfish monocyte-like cells, sorted based on size and internal complexity. Sorted monocytes were treated with either 10ng/mL of rgCSF-1 or cell conditioned medium. The percentage of total events (25000) with the standard error associated with mean percentage value that occur in the macrophage gate is indicated in the bottom right corner of each plot. This is a flow cytometry dot plot representing monocyte subpopulation obtained from an individual fish. This analysis was done using cell cultures established from individual fish (n=8) and the results of a representative experiment are shown.

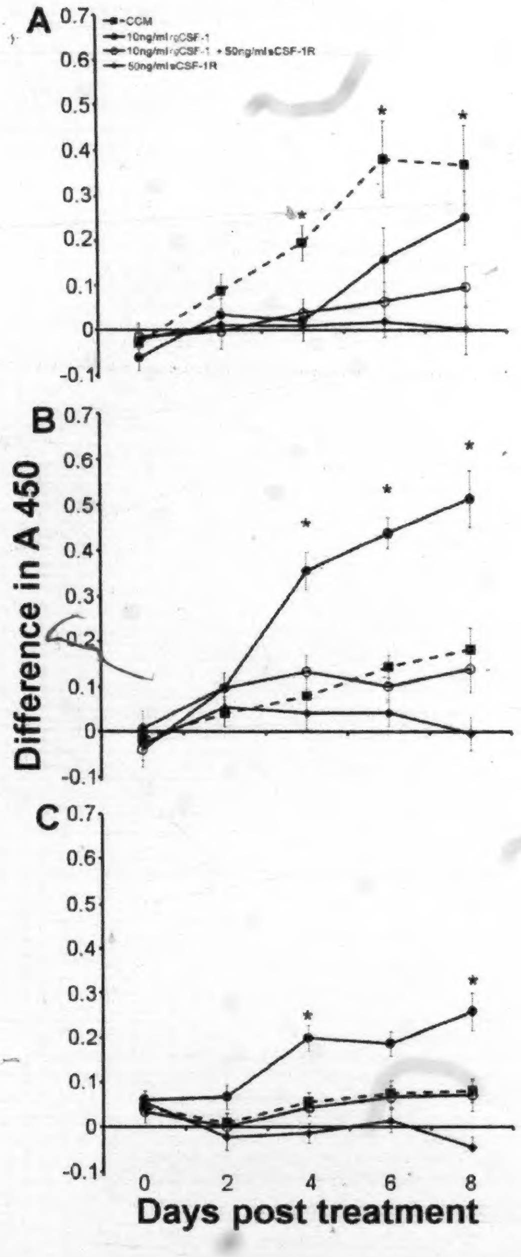


Figure 4.5 rgCSF-1 induces monocyte proliferation that is abrogated by sCSF-1R.The proliferative response of sorted goldfish progenitor cells (A), monocytes (B) and macrophages (C) treated for 8 days with ether cell conditioned medium (CCM), 10 ng/mL of rgCSF-1, 10 ng/mL of rgCSF-1 pre-incubated with 50 ng/mL of sCSF-1R, or 50 ng/mL of sCSF-1R alone. The values for non-treated cells were subtracted from experimental values to control for production of endogenous growth factors by the cells. Statistical significancewas determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=8).

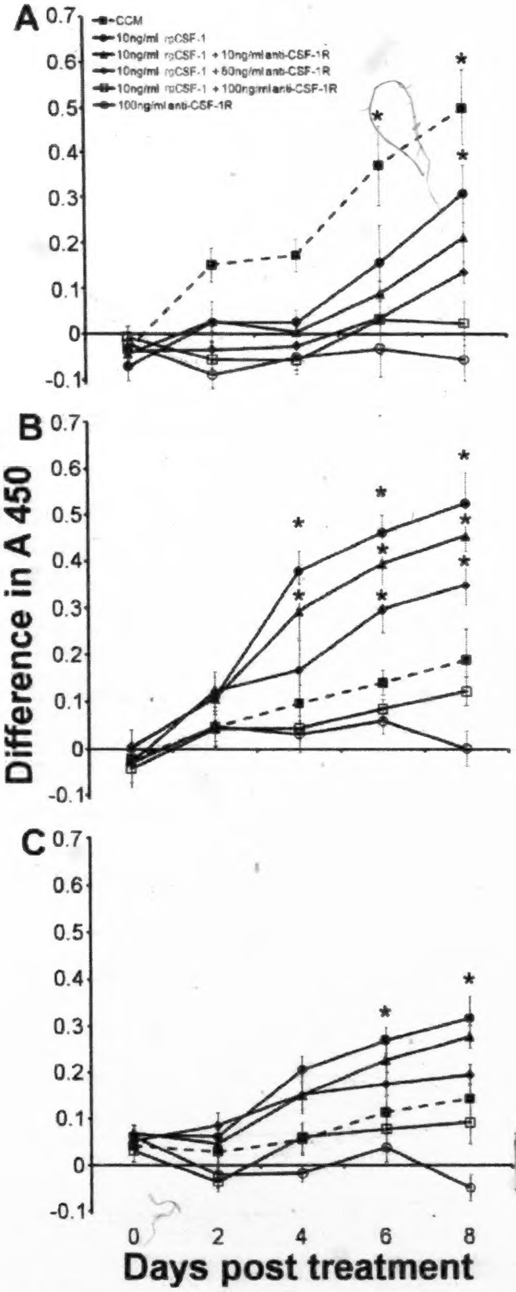


Figure 4.6 rgCSF-1 induces monocyte proliferation that is abrogated by the anti-CSF-1R antibody. The proliferative response of sorted goldfish progenitor cells (A), monocytes (B) and macrophages (C) treated for 8 days with cell conditioned medium, 10 ng/mL of rgCSF-1, 10 ng/mL of rgCSF-1 pre-incubated with 10 ng/mL, 50 ng/mL or 100 ng/mL of anti-CSF-1R antibody, or 100 ng/mL of anti-CSF-1R antibody alone. The values for non-treated cells were subtracted from experimental values to control for production of endogenous growth factors by the cells. Statistical significancewas determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=8).

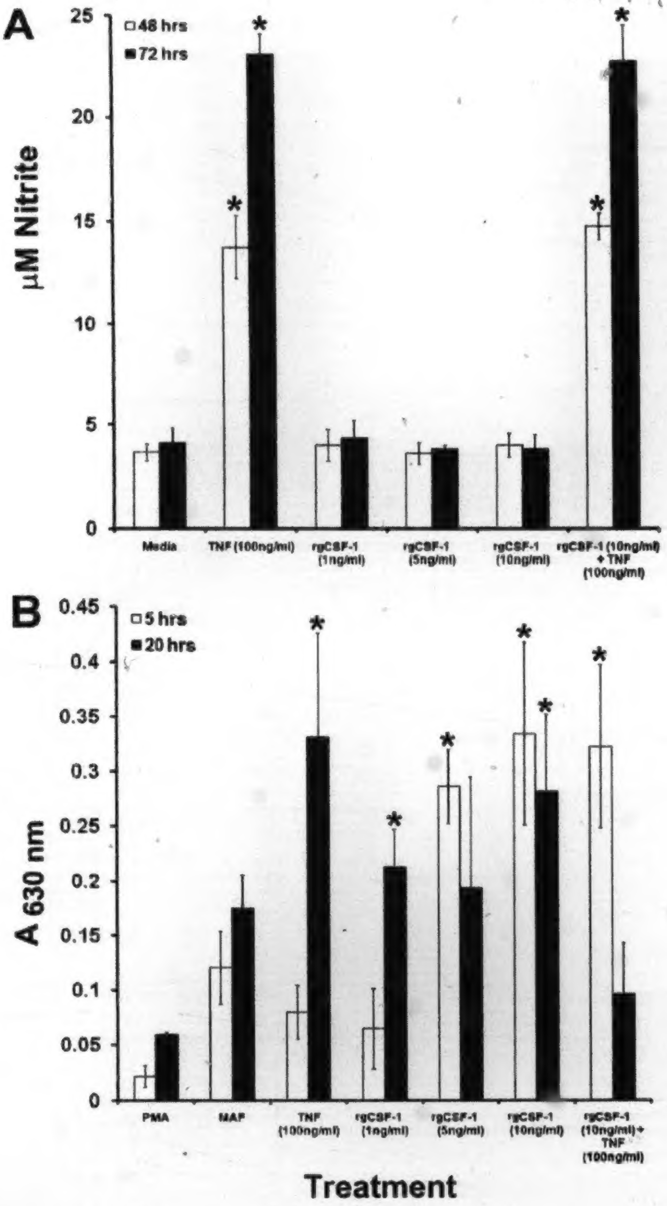


Figure 4.7 rgCSF-1 induces production of reactive nitrogen and oxygen intermediates by goldfish macrophages. Induction of reactive nitrogen intermediate production as measured by the presence of nitrite in culture supernatants determined by the Griess reaction. Nitrite production induced by rgCSF-1 after 48 or 72 hours post treatment was compared to medium (negative) and goldfish rTNF-α (positive) controls (A). Induction of reactive oxygen intermediates measured by the reduction of NBT. Induction of ROI by rgCSF-1 after 5 or 20 hours post treatment was compared to medium (negative) and MAF or goldfish rTNF-α (positive) controls (B).Each bar represents the mean ± SEM (n=6). Statistical significancewas determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*).

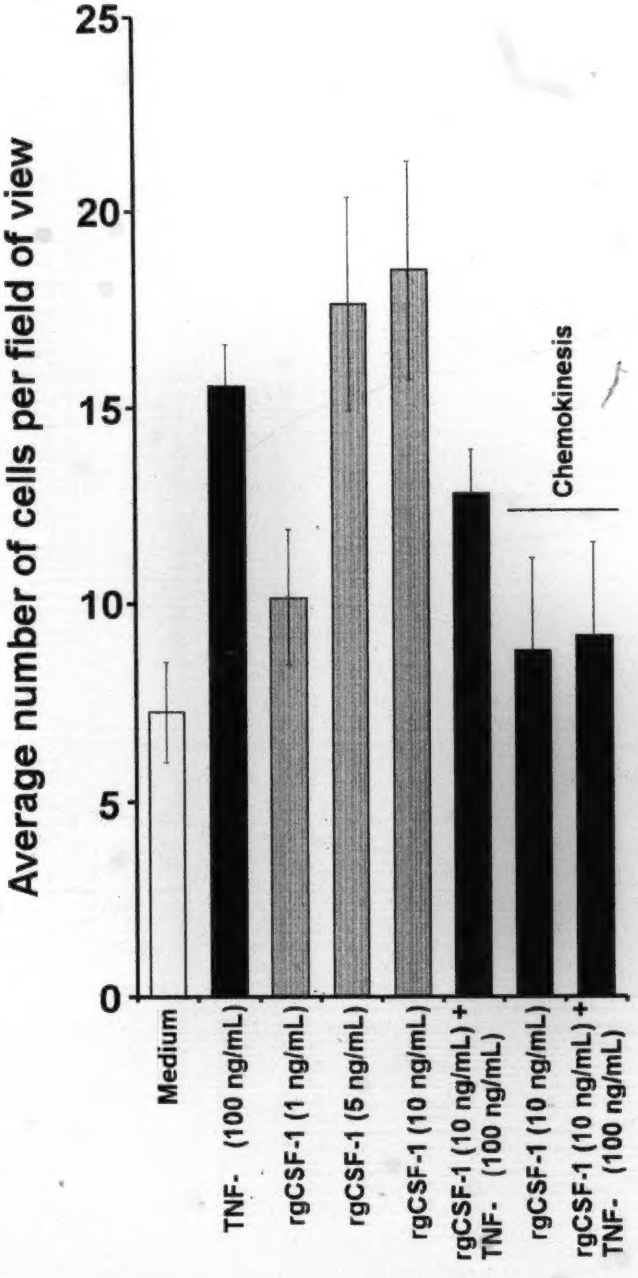


Figure 4.8 Induction of chemotactic response of goldfish primary kidney macrophages by rgCSF-1. Cell migration is shown as the average number of cells viewed under the 100X objective for each experimental group (average number of cells/view of 20 randomly selected fields). Migration induced by

rgCSF-1 is compared to medium (negative) or goldfish rTNF-α (positive) controls. Chemokinetic activity was assessed to rule out random migration induced by rgCSF-1. Each treatment was repeated using cell cultures established from 4 individual fish (n=4). Statistical significancewas determined using one-way ANOVA and Tukey post hoc analysis, ($P< 0.05$) is denoted with (*).

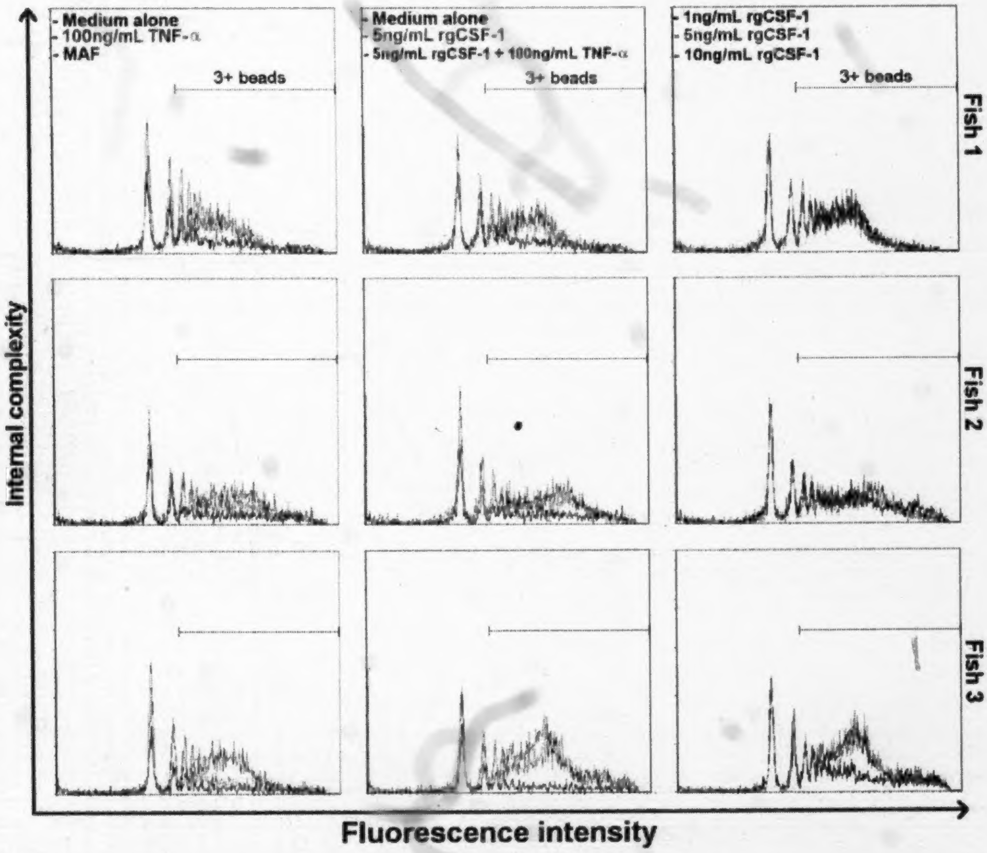


Figure 4.9 rgCSF-1 enhances phagocytosis of goldfish primary kidney macrophages. Three independent experiments are shown using cell cultures obtained from individual fish; controls (medium alone, MAF and TNF-α) on the left, effects of rgCSF-1 added in combination with TNF-α (centre), and the effects of rgCSF-1 at increasing concentrations (right). Significant increase in phagocytic activity is indicated by the gated area which represents cells that have phagocytosed 3 or more fluorescent beads.

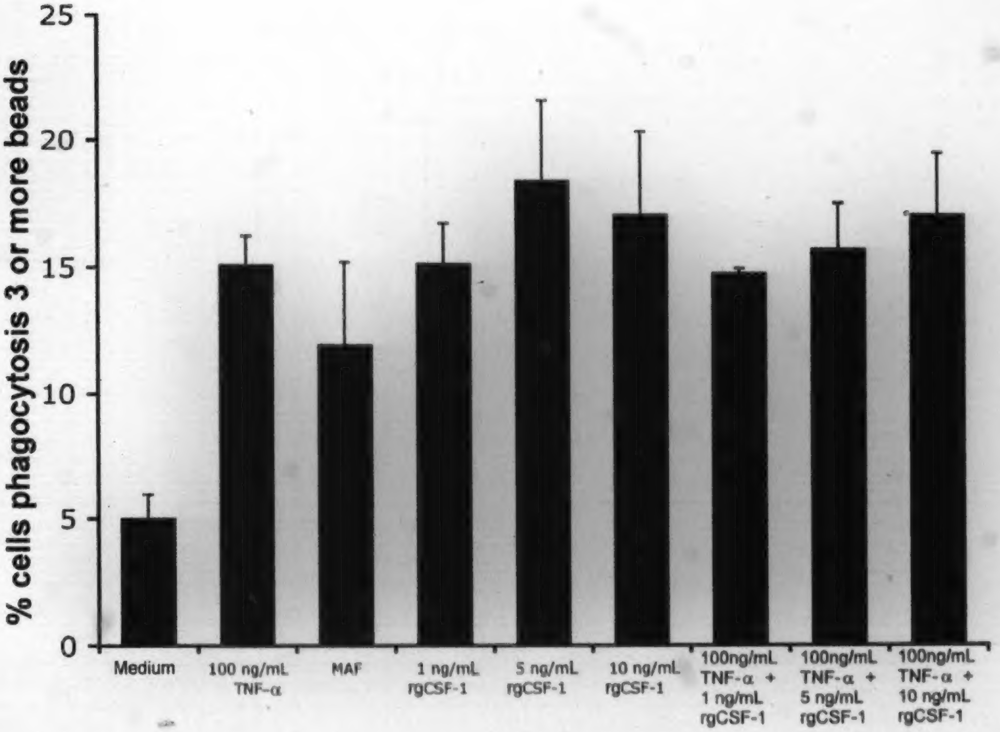


Figure 4.10 The percent of goldfish primary kidney macrophages that engulfed 3 or more fluorescent latex beads after treatment with rgCSF-1.Treatments of rgCSF-1 were compared to medium alone, TNF-α and MAF controls. The affect of rgCSF-1 addition in combination with TNF-α was also assessed. Bars represent the mean % of phagocytic cells with 3 or more beads ± SEM (n=3). All treatments were found to significantly ($P<0.05$) enhance phagocytosis over the medium control was determined using one-way ANOVA and Tukey post hoc analysis,.

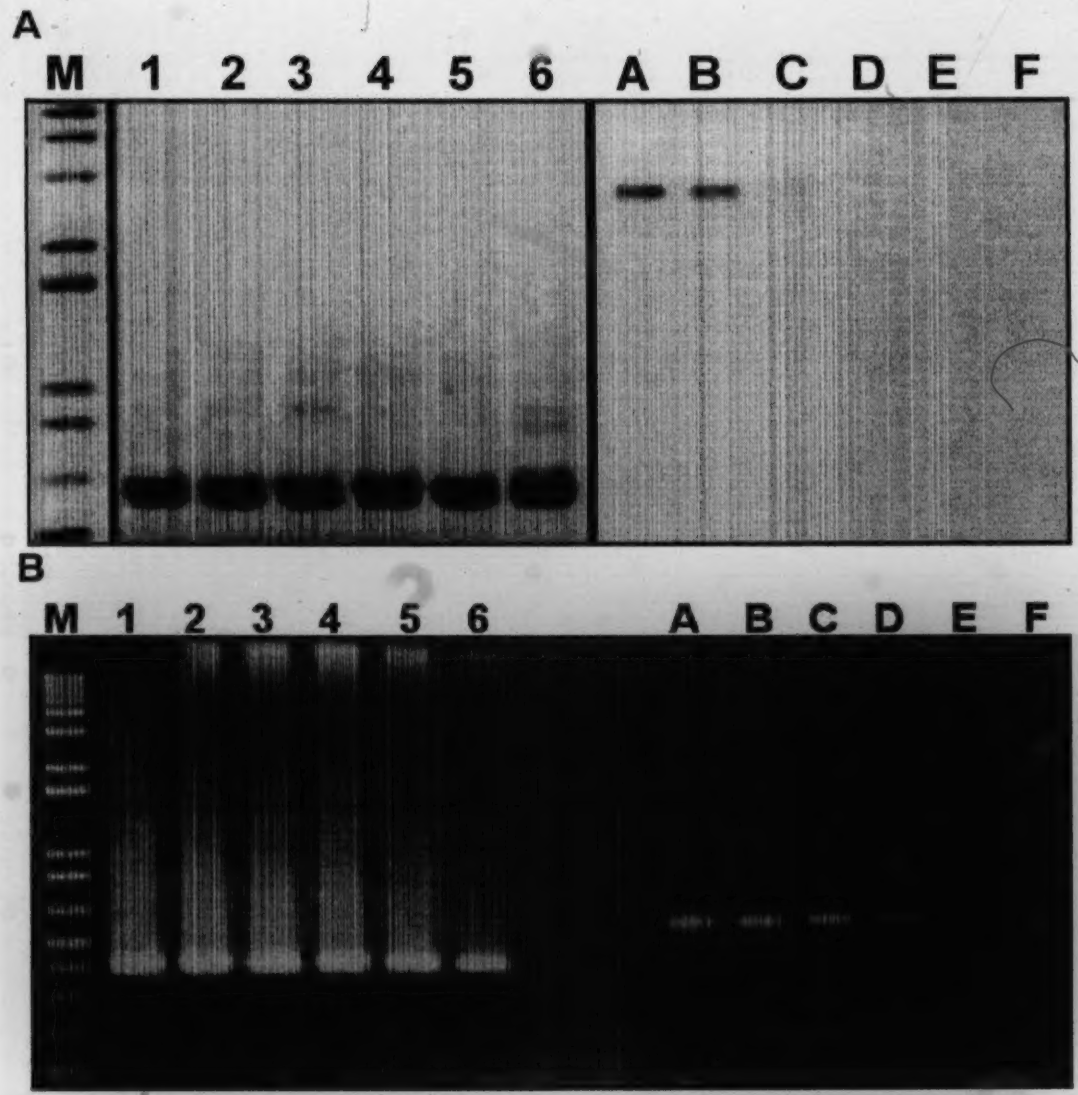


Figure 4.11 RNAi induced knockdown of CSF-1R message. Non-isotopic northern blot (A) and RT-PCR (B), showing the RN[illegible]induced knockdown of the CSF-1R mRNA message. M=size marker, 1, 2, 3, 4, 5, and 6 represent β-actin expression after 18 hours of exposure to none, 1nM, 2.5nM, 5nM, 7.5nM and 10nMof CSF-1R-specific dsRNA oligonucleotides respectively (A and B). A, B, C, D, E, and F represent CSF-1R message after none, 1nM, 2.5nM, 5nM, 7.5nM and 10nM of CSF-1R-specific dsRNA oligonucleotides, respectively (A and B). Both A and B are a representative blot/gel of three independent experiments that were performed.

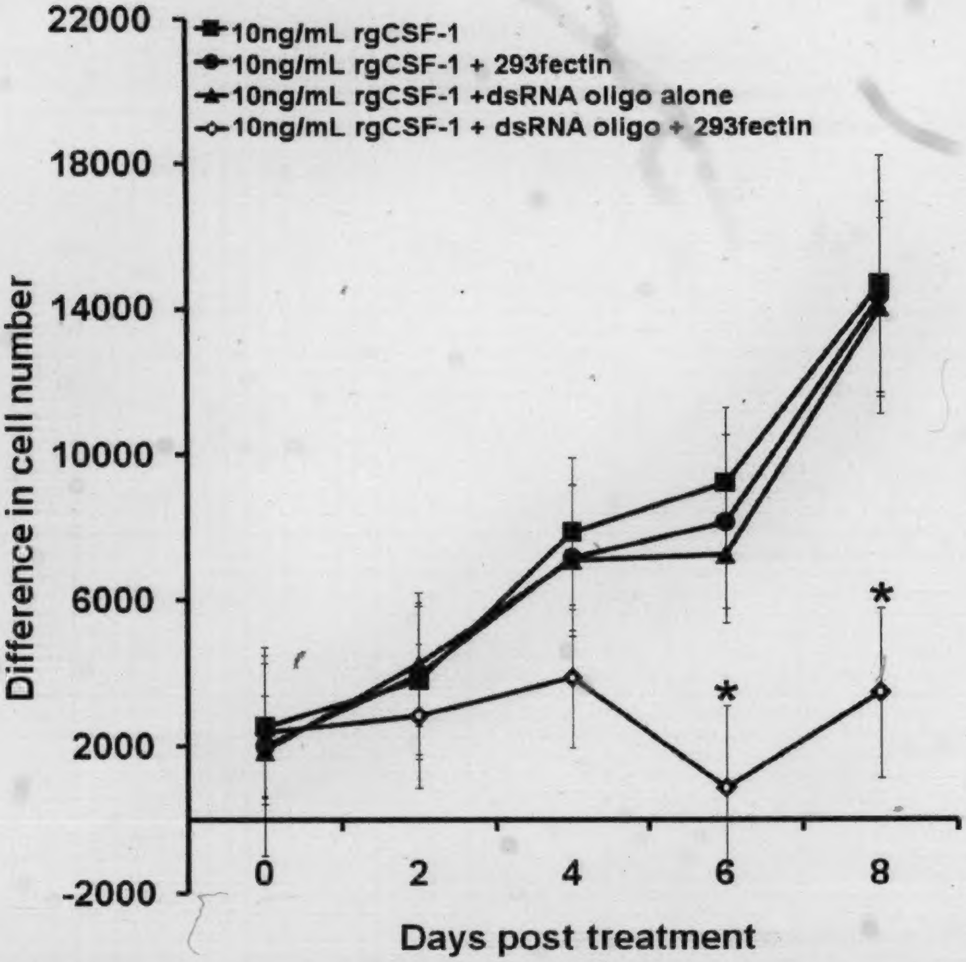


Figure 4.12 rgCSF-1 induces proliferation of goldfish primary macrophages that is abrogated by RNAi-mediated knockdown of the CSF-1R.Cell number was determined every 2 days until day 8 post treatment. Proliferation induced by rgCSF-1 was compared to cells treated with rgCSF-1 + 293fectin alone, and rgCSF-1 + CSF-1R-specific dsRNA oligonucleotides alone, to confirm the two components of the RNAi knockdown did not have independent affects. Values for medium controls were subtracted from each treatment to account for production of endogenous growth factors by the cells. Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=8).

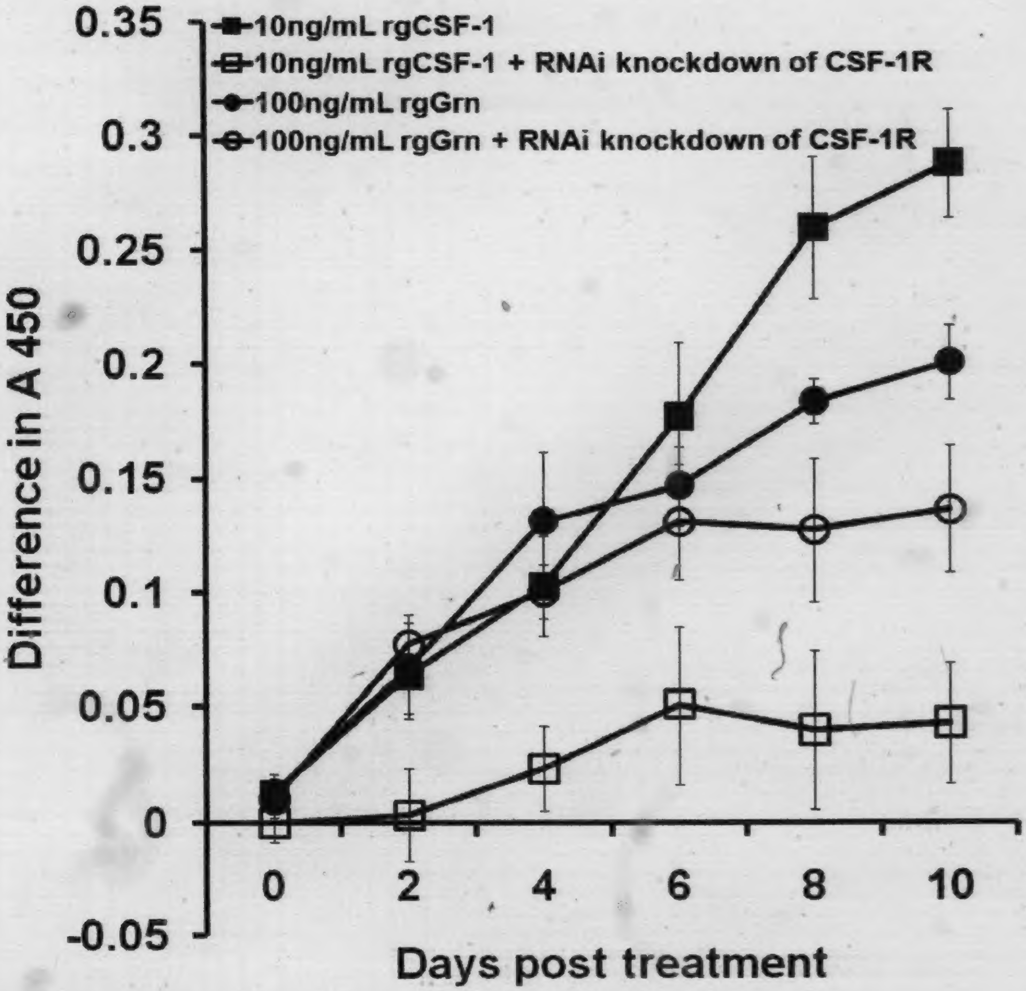


Figure 4.13 RNAi mediated knockdown of the CSF-1R does not abrogate granulin-mediated proliferation in goldfish PKM cultures. Knockdown of CSF-1R abrogated rgCSF-1-induced proliferation, however it did not significantly affect proliferation induced by 100 ng/mL of rgGrn. Values for medium controls were subtracted from each treatment to account for production of endogenous growth factors by the cells. Each point on the graphs represents mean ± SEM (n=6).

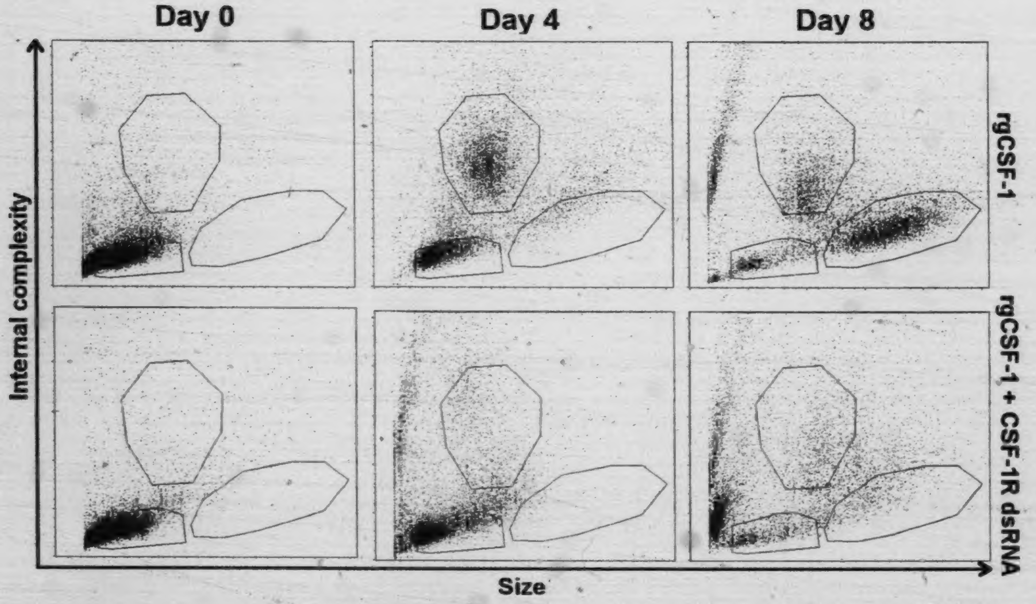


Figure 4.14 RNAi knockdown of CSF-1R inhibits CSF-1-induced monocyte differentiation. Flow cytometric dot plot showing the differentiation of moncytes/macrophages from progenitor cells (Day 0 cells isolated from goldfish kidney). Treatment of day 0 cultures with rgCSF-1 induces the differentiation of monocytes and macrophages by day 4 post treatment. Pre-treatment of the cultures with CSF-1R-specific dsRNA to mediate RNAi knockdown of the CSF-1R and then treatment of these cells with rgCSF-1 results in an abrogation of rgCSF-1-induced differentiation. Dot plots are representative of 4 independent experiments that were performed.

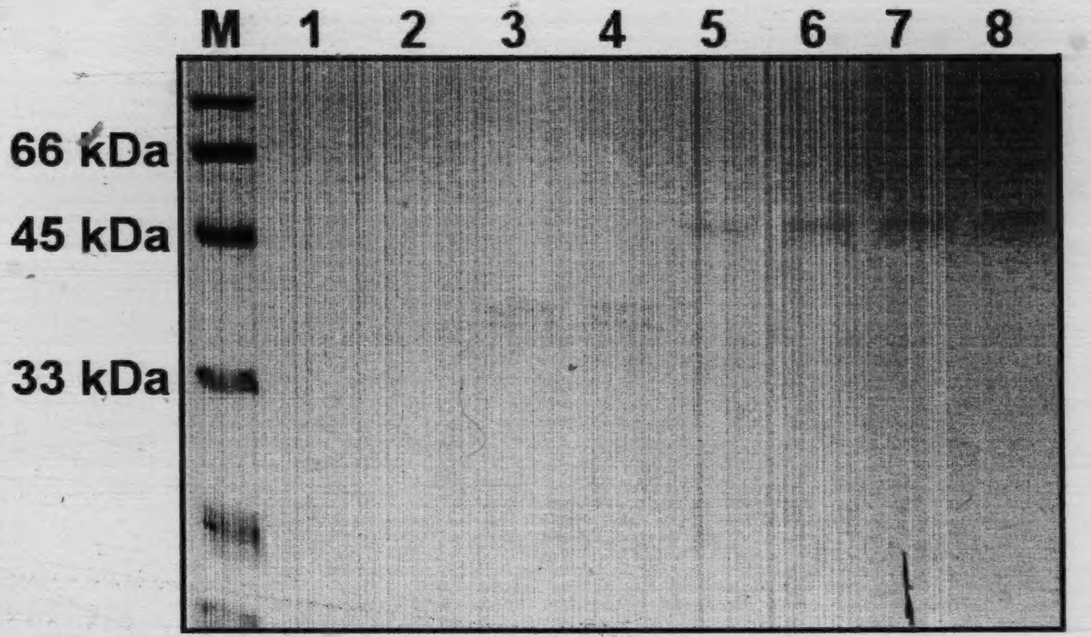


Figure 4.15 Far Western blot showing the presence of native goldfish CSF-1 in the supernatants of primary kidney macrophage cultures. The Western blot was initially probed with 10 ng/mL, 25 ng/mL, 50 ng/mL or 100 ng/mL of soluble CSF-1R (sCSF-1R) and then the anti-CSF-1R antibody was applied to detect the location of the receptor or where it bound. Lanes 1-4 represent 10, 25, 50 and 100 ng/mL sCSF-1R, respectively ran as controls, lanes 5-8 represent 10X concentrated cell conditioned supernatants (albumin removed) probed with 10, 25, 50 and 100 ng/mL of sCSF-1R respectively. The supernatants were obtained from day 6 cultures.

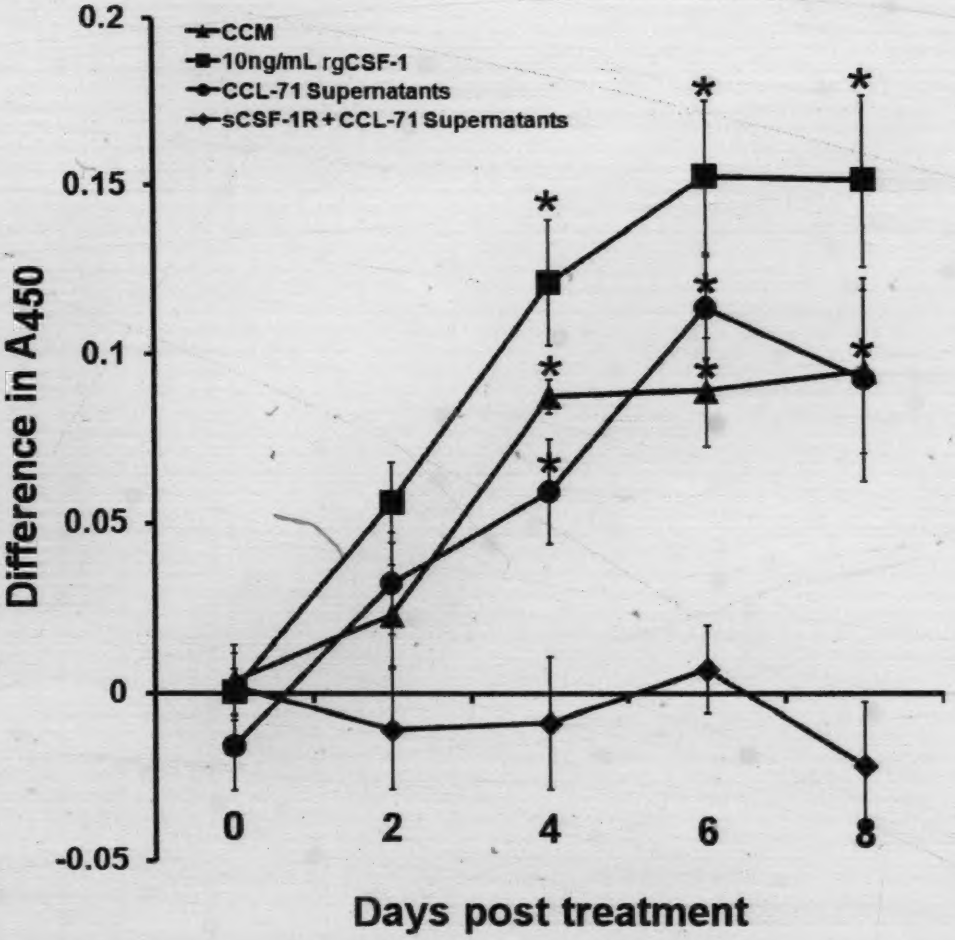


Figure 4.16 CCL-71 goldfish fibroblast culture supernatants induce proliferation of primary kidney macrophage cultures. Proliferation induced by cell conditioned supernatants (CCM) from CCL-71 goldfish fibroblast cell line. CCL-71 CCM induced proliferation was compared to that induced by rgCSF-1 and primary kidney macrophage CCM. The proliferative response of goldfish cultures to CCL-71 CCM was CSF-1-mediated as indicated by the abrogation of CCL-71 CCM induced proliferation after addition of the sCSF-1R. Medium controls were subtracted from all treatments to account for endogenous growth factor production. Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=6).

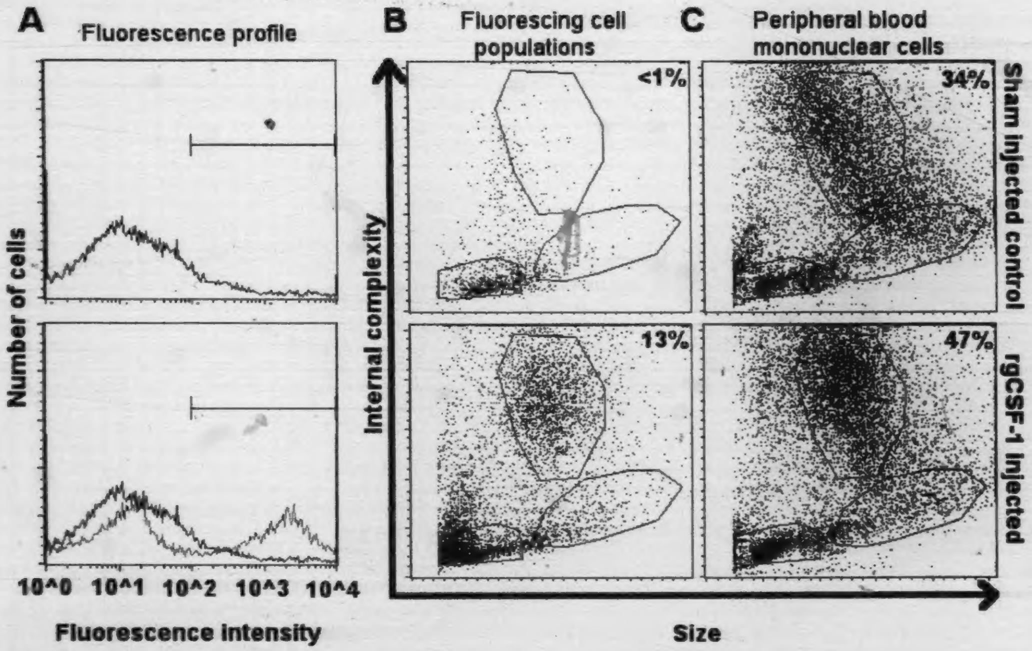


Figure 4.17 Administration of rgCSF-1*in vivo* increases circulating blood monocyte cell pool. *In vivo* proliferation assay showing the effects of injection of rgCSF-1 on circulating mononuclear cells in blood compared to a sham-injected control. Cells that have undergone proliferation after treatment were labelled with fluorescent BrdU and detected using flow cytometry. The BrdU-labelled population (A) was further analysed based on size and internal complexity (B), where the percentages of total events analyzed (out of 25,000) that are fluorescing in the monocyte gate are indicated . The whole blood analysis based on size and internal complexity is shown in (C) where the percentages of cells out of the total cell pool analysed that are monocytes are indicated.

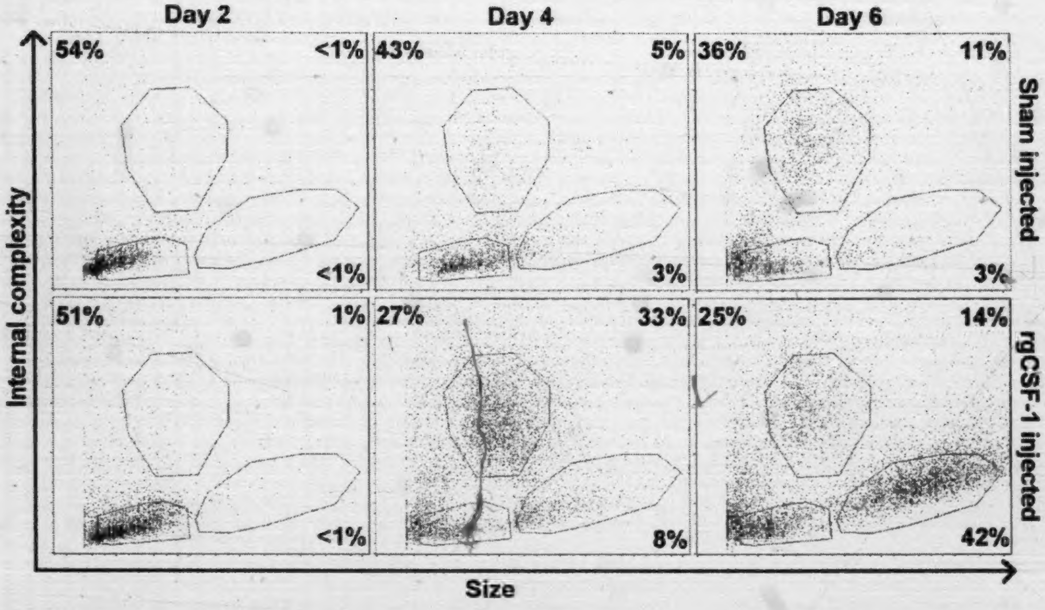


Figure 4.18 Administration of approximately 10 ng/mL rgCSF-1 to goldfish increases the number primary kidney monocytes and macrophages in cultures established from treated fish. Flow cytometric profiles of primary kidney cultures derived from fish injected with BrdU to label proliferating cells. These dot plots show the fluorescing cells which have proliferated since labelling and treatment with either a sham 1X PBS injection, or rgCSF-1 injection. Values in the top left, top right and bottom right of each profile indicate the percent of fluorescing cells in the progentior, monocyte and macrophage gates, respectively, out of the 25,000 total events analyzed.

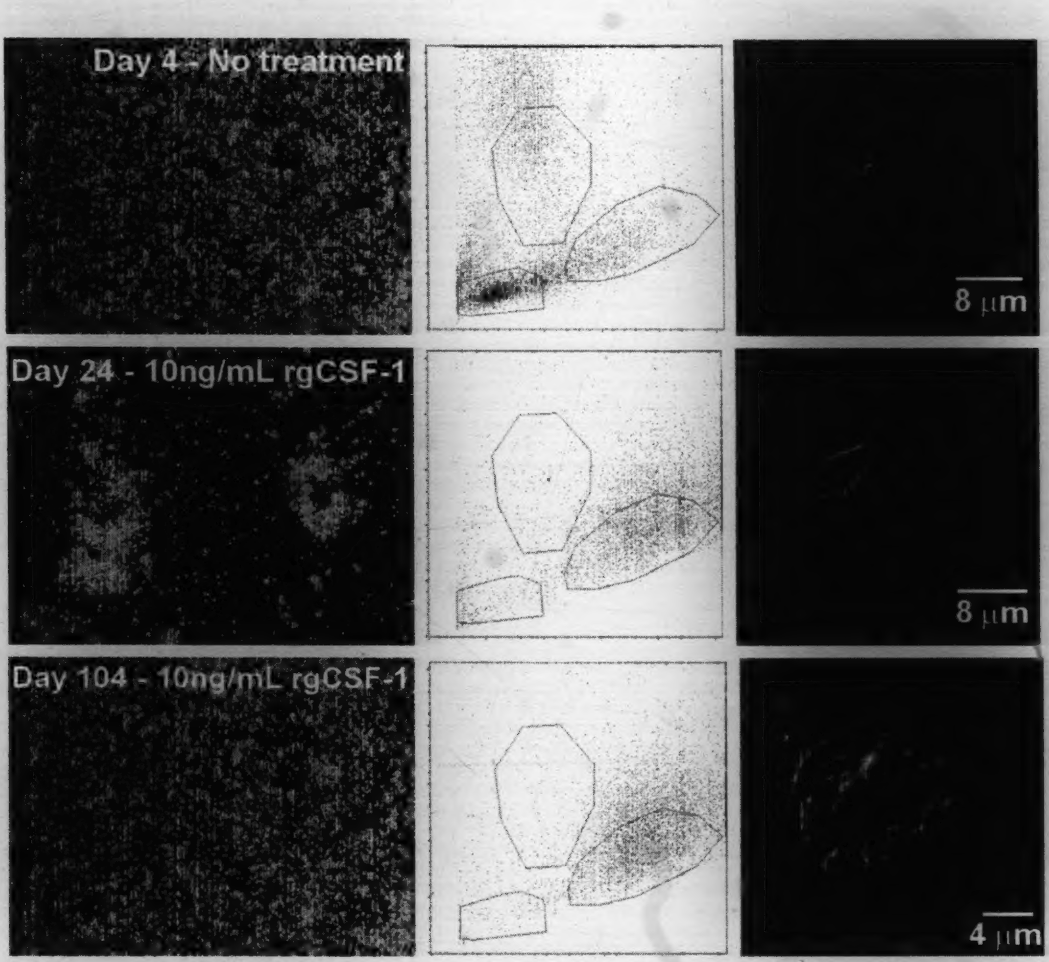

Day 4 - No treatment
8 µm
Day 24 - 10ng/mL rgCSF-1
8 µm
Day 104 - 10ng/mL rgCSF-1
4 µm

Figure 4.19 Analysis of long-term primary kidney macrophage cultures supplemented with rgCSF-1. The left panels show inverted microscope images at 10X magnification. The centre panel shows the flow cytometer profiles (based on size and internal complexity)of these cultures on the days post cultivation indicated. The left panel is a representative DIC image of individual cells from the cultures.

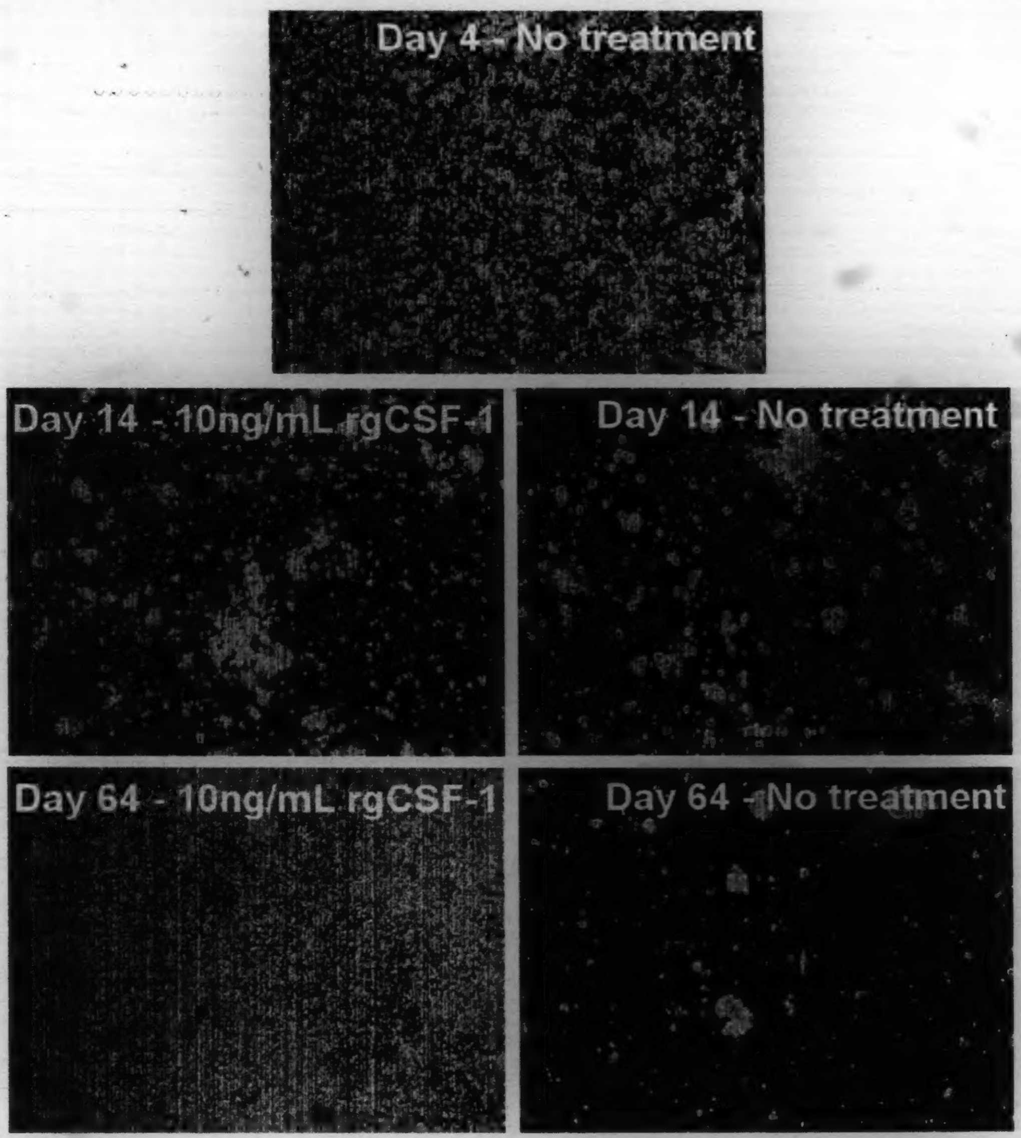

Day 4 - No treatment
Day 14 - 10ng/mL rgCSF-1
Day 14 - No treatment
Day 64 - 10ng/mL rgCSF-1
Day 64 - No treatment

Figure 4.20 Weekly supplementation with rgCSF-1 induces prolonged proliferative response and survival of goldfish primary kidney macrophages *in vitro*. Inverted microscope images taken at 10X magnification showing the differences in culture morphology between rgCSF-1- supplemented cultures and non-treated cultures on the days indicated.

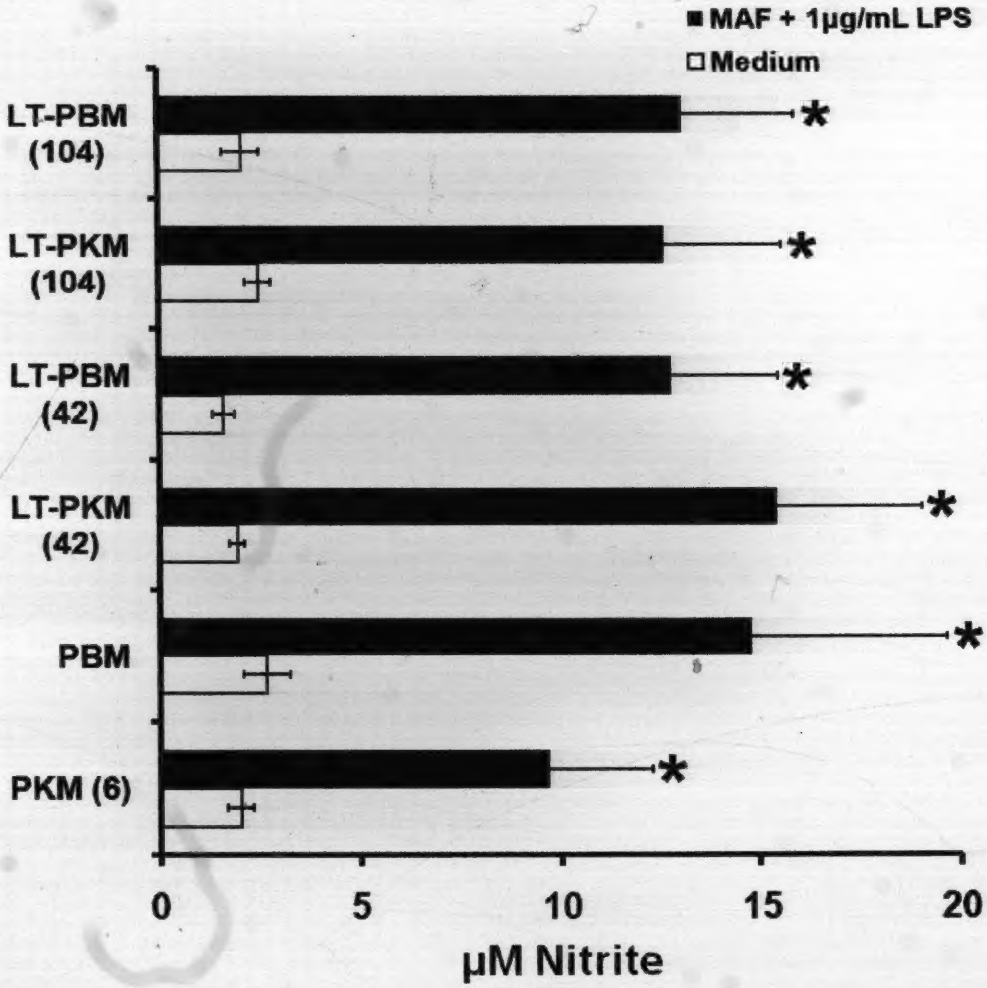


Figure 4.21 Long term primary kidney and blood mononuclear cells are able to produce reactive nitrogen intermediates. Induction of reactive nitrogen intermediate production as measured by the presence of nitrite in culture supernatants was determined by the Griess reaction. Nitrite production induced by macrophage activation factor (MAF) and 1 µg/mL LPS after 72 hours post treatment was compared to medium (negative control). Each bar represents the mean ± SEM (n=6). Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*).

Chapter 5

Molecular and Functional Characterization of Granulin: A novel growth factor of goldfish macrophages[1]

5.1 Introduction

Since their discovery [265, 297], granulins have been shown to promote proliferation of different mammalian cell types [244, 261, 469]. First identified as small (6 kDa) peptides, granulins are produced by the proteolysis of a larger precursor molecule by leukocyte derived elastase [262, 265, 297, 470]. The larger granulin precursor is known by several names, including granulin/epithelin precursor [273], proepithelin [263], acrogranin [264], PC cell-derived growth factor (PCDGF) [245] and progranulin. Granulins are characterized by a unique 12 cysteine motif that is arranged in 4 β-hairpins, stacked one upon another in a helical formation connected by disulphide bonds [471-473]. The molecular structure of granulin is unique although there are some structural similarities between granulin and the epidermal growth factor/transforming growth factor-alpha family of molecules [472]. Along with the currently identified mammalian granulins identified in human [265, 470], rat [297, 471], mouse [264, 474] and horse [475], granulin-like proteins have also been identified in a number of non-mammalian organisms including the nematode *Caenorhabditis elegans* [476], the nervous system of the locust [291], the mussel *Patinopecten yessoensis* [290], the marine worm *Hediste*

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[1] A version of this chapter has been published:
Hanington et al. 2006. A novel hematopoietic granulin induces proliferation of goldfish (*Carassius auratus* L.) macrophages. Journal of Biological Chemistry. 281: 9963-70.

diversicolor [477], and bony fish [478-480]. Interestingly, granulin-like motifs have even been identified in multiple thiol protease gene sequences from plants [481, 482].

The mammalian progranulin genes exhibits ubiquitous expression in tissues [263, 264, 288, 289, 483, 484], and a number of epithelial and hematopoietic cell lines [289, 483], as well as different neoplastic cells [248-250, 485-488]. Progranulin is primarily expressed in epithelial cells that exhibit rapid turnover, such as the cells found in the intestinal microvilli [289]. It is also expressed in cells of the immune and nervous systems [294, 478, 479]. Almost all of the known granulin genes that encode for functional peptides are progranulin genes. However, granulin-like transcripts have been identified in the zebrafish that appear to encode for one and one half granulin cysteine motifs (AF273479, AF273480). Although a number of granulin genes have been identified in lower vertebrates and invertebrates, many of the peptides encoded by these genes have yet to be functionally characterized.

The results of studies on progranulin and its cleavage products have described diverse functions for the molecule. Progranulin has been shown to be involved in different stages of embryonic development [254, 489-491], sexual differentiation of the rat brain via actions on the ventromedial hypothalamus [256, 492], and as a possible trigger for rat copulatory behaviour [493]. Progranulin induces proliferation in embryonic fibroblasts (R- cells) from mice unable to produce functional insulin-like growth factor-1 receptor (IGF-1). This feature of progranulin makes it unique among known growth factors as it is the only growth factor that is able to induce proliferation of R- cells in the absence of IGF-1 and platelet-derived growth factor [494].

The second messenger pathways used by progranulin to induce cellular proliferation have been characterized. Progranulin signals through the p44/42 mitogen-activated protein kinase (MAP kinase) and the phosphatidylinositol 3-kinase (PI3 kinase) pathways and induces the expression of cyclin D1 and cyclin B. Interestingly, these pathways, are also involved in the signalling of IGF-1 and thus, may be the reason why progranulin can act in place of IGF-1 [273, 280, 281]. In addition, progranulin has been implicated in the inflammatory response as it can induce cellular migration during wound healing [252, 261, 274]. Although the multi-functional nature of the progranulin is well characterized, a receptor for progranulin or its cleavage products has yet to be identified.

In this chapter, I describe the molecular and functional analyses of a unique granulin gene identified in the goldfish (*Carassius auratus*) by differential cross screening of goldfish macrophage cDNA libraries.

5.2 Experimental design

5.2.1 Quantitative PCR analysis of granulin expression in different tissues and sorted cell populations

The relative expression of goldfish granulin in relation to β-actin was assessed in the kidney, spleen, liver, heart, brain, gill, and intestine. Thermocycling parameters were as follows: 95°C for 2min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min.

5.2.2 Prokaryotic expression of goldfish granulin

Goldfish granulin was expressed using the pET SUMO prokaryotic protein expression system. PCR amplification of the protein expression construct insert was performed as follows: 7 μL of the granulin clone template was added to 76 μL ddH2O, dNTPs (0.2 μL of each dATP, dCTP, dGTP, dTTP 100 mM solutions), 10x PCR buffer (10 μL of 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15mM $MgCl_2$, 0.01% (w/v) gelatin), expression primers (2.4 μL of each 20 μM solution) and a 15:1 ratio of Taq:Pfu DNA polymerases (1 μL of 5 U μL-L solution). PCR amplification was conducted in an Eppendorf Mastercycler Gradient thermal cycler. The amplification program consisted of a 3.5 min hot-start at 94°C, followed by 25 cycles of 94°C for 20 sec, 55°C for 20 sec, 72°C for 2 min, and a final elongation step of 72°C for 7 minutes.

5.2.3 Measurement of macrophage proliferation induced by purified granulin

Cells suspended in 50 μL of complete culture medium were seeded in 96-well plates and treated with 50 μL of 250 ng/mL, 100 ng/mL, 10 ng/mL, or 1 ng/mL granulin dissolved in incomplete cell culture medium and incubated for 52 hours at 20°C. BrdU labelling reagent was added at 15 μM (final concentration) and cells were incubated for an additional 24 hours at 20°C. The reaction was developed according to the manufacturer's specifications and optical densities determined at 450 – 640 nm using a microplate reader. In control experiments the colorimetric reaction was found to be directly proportional to the number of proliferating PKM in culture.

To determine whether the anti-rgGrn antibody was able to block the proliferation-inducing effects of rgGrn serial dilution series of the anti-rgGrn antibody were prepared and mixed with 5 μM samples of goldfish granulin prior to addition to a 96-well plate containing 1 x 10^4cells/well. The BrdU proliferation assay was done as described in chapter 3.

5.3 Results

5.3.1 *Molecular analysis of goldfish granulin*

The most common transcript identified in differential cross screening of proliferative and senescence phase goldfish macrophage cDNA libraries was granulin (Fig. 5.1) Thirty one partial granulin-like transcripts were identified, and all exhibited higher expression in proliferating macrophages. All of the transcripts were sequenced and were found to be identical. The fully sequenced cDNA transcript of goldfish granulin was 947 nucleotides in length with an open reading frame of 477 nucleotides. The predicted protein was 159 amino acids long and had 18 conserved cysteine residues, 12 of which represent a full granulin cysteine motif common for all known granulin proteins. The remaining 6 cysteine residues made up one half of this motif (Fig 5.2).

The predicted goldfish granulin protein had conserved amino acids found in granulins spanning the metazoans. Granulins have been identified in mammals, fish, insects, bivalves and nematodes. The amino acid sequence alignment of goldfish granulin and other known fish granulins of carp, zebrafish and goldfish show highly conserved cysteine rich motifs (Fig. 5.3). Goldfish granulin was most similar to carp granulin-3, with an amino acid identity of 56%. Of all the granulins analyzed, goldfish granulin shared the highest identity with other fish granulins (Fig. 5.4), which was supported by phylogenetic analysis that grouped goldfish granulin in close proximity to carp granulin-2 and granulin-3 (Fig. 5.4). Phylogenetic analysis also suggested that the granulins of fish all shared distinct features that separated them from the granulins of mammals. Although the granulin proteins identified in carp and from goldfish intestine have no corresponding mRNA transcript sequences, zebrafish granulin-1, granulin-2 and zebrafish hybrid granulin, had similar transcript organization to that of goldfish granulin.

The expression of goldfish granulin transcript was analyzed by Northern blot, RT-PCR, and real time PCR. Analysis of transcript expression in the heart, brain, spleen, kidney, gill, liver and intestine revealed that goldfish granulin was expressed primarily in the kidney and the spleen (Fig. 5.5 A and B). Real time PCR and RT-PCR analyses of granulin expression were also done using non-activated and activated macrophages, and sorted goldfish macrophage subpopulations. Goldfish granulin expression was up regulated in activated macrophages compared to non-activated controls (Fig. 5.6 A and B). Interestingly, goldfish granulin was primarily expressed in the monocyte subpopulation, with lower expression evident in mature macrophages and the early progenitor cells (Fig. 5.7 C and D).

5.3.2 *Functional analysis of goldfish granulin*

To examine the effect(s) of granulin on fish macrophage development *in vitro*, we generated recombinant goldfish granulin (rgGrn) using a prokaryotic expression system. The recombinant protein was used to generate an affinity-purified rabbit IgG. The anti-rgGrn IgG recognized rgfGrn (Fig. 5.7 E), as well as native goldfish granulin in goldfish macrophage culture supernatants, indicating that the molecule was secreted by actively growing PKM.

The ability of rgGrn to induce a proliferative response of goldfish macrophages was tested by adding different amounts (5 ng/mL to 100 ng/mL) of the rgGrn to newly established cultures of sorted early progenitor cells, monocytes and mature macrophages. The proliferation assay was done for triplicate PKM cultures established from individual fish, (n=8). All optical density values were normalized to those of cells alone control. The enhancement of cellular proliferation in primary kidney macrophage cultures was noticeable when compared to that of cultures treated with CCM or vector controls, as early as 2 days post treatment with higher concentrations of rgGrn inducing a greater proliferative response in sorted progenitor cells than CCM controls (Fig. 5.7D). The effects of rgfGrn on the proliferation of sorted cell populations was variable: rgGrn induced significant proliferative response of the progenitor cells (3 times higher than CCM by day 8 of cultivation) (Fig. 5.7A), and lower but significant proliferation of monocytes (1.6 times higher than CCM by day 8 of cultivation) (Fig. 5.7B). In contrast, the mature macrophage subpopulation did not proliferate in the presence of rgfGrn (Fig. 5.7C). The addition of anti-rgGrn IgG abrogated the proliferative response of macrophages in a dose-dependent manner (Fig. 5.7F). No increase in proliferation of cultured macrophages was observed in the presence of the vector control or the anti-rgGrn antibody alone (Fig. 5.7).

5.4 Discussion

This chapter details the work on the molecular and functional characterization of a novel granulin-like gene of the goldfish. Granulin was the most common gene identified during cross-screening of the goldfish macrophage proliferative and senescence phase libraries [45]. Northern blot analysis as well as RACE PCR confirmed that this transcript was unique and that it did not share a high degree of identity with any other granulin genes that may be present in the goldfish. Goldfish granulin shared high sequence identity with currently identified granulin genes; however, it was much smaller than the mammalian progranulin genes, consisting of only one and one-half of the 12 cysteine motif characterizing all granulins. Northern blot analyses revealed that this granulin gene was highly expressed in the hematopoietic tissues of the goldfish. Quantitative and RT-PCR analyses showed that this expression pattern was likely due to the presence of monocyte/ macrophage-like cells in these tissues. The goldfish granulin characterized in this thesis was found to be differentially expressed in different macrophage subpopulations, and it induced a dose-dependent proliferation of PKM *in vitro*.

Granulins were first purified from the extracts of human inflammatory cell exudates, and from rat bone marrow [265]. To date, seven granulin peptides (A to G) have been characterized [263, 264, 288, 474, 483], and it has been shown that they are generated following proteolytic cleavage of progranulin [262]. The main difference between goldfish granulin and mammalian progranulin is that this goldfish granulin gene encodes for a much smaller protein (159 amino acids). Furthermore, in contrast to the ubiquitous expression of mammalian progranulin, the expression of this goldfish granulin transcript was confined to the fish hematopoietic tissues (kidney and spleen). The presence of three granulin proteins in hematopoietic tissues of the carp has been reported; granulin-1 which was found to be mainly in extracts of the spleen, and granulin 1, granulin-2 and granulin-3 that were present in extracts of the head kidney [478]. Furthermore, antibodies generated against carp granulin-1 appeared to recognize the cells of mononuclear phagocyte lineage in the head kidney of carp [478, 479]. Sequence data of zebrafish granulins (GenBank AF273479 and AF273480) suggest that this fish species possesses two genes that encode granulin proteins. The zebrafish granulins have yet to be functionally characterized. Similar to the goldfish granulin identified in my study, zebrafish granulin 1 and granulin-2 also have one and one half cysteine repeats, suggesting that they may be the possible orthologs to goldfish granulin. In addition, this suggests that there are likely more goldfish granulin molecules and possibly goldfish pro-granulin as well.

We previously demonstrated that primary goldfish macrophages, unlike mammalian macrophages, grow spontaneously *in vitro* and that they secrete their own growth factor(s) [44, 53]. The differentiation of goldfish monocytes and macrophages from progenitor cells occurred via two pathways; the classical differentiation pathway of progenitor to monocyte and then to macrophage, as well as a unique pathway where progenitor cells differentiated directly into macrophages [44, 53]. Since the expression of goldfish granulin was upregulated in proliferating PKM, we hypothesized that granulin molecule may play an important role in control of cell proliferation. Recombinant granulin induced a significant and dose-dependent proliferative response in early progenitor and monocyte subpopulations of PKM *in vitro*, indicating that this molecule may regulate goldfish macrophage hematopoiesis.

Much work has been done to define the role of progranulin in mammalian model systems. Progranulin was shown to be involved in different stages of embryonic development [253, 254], sexual differentiation of the rat brain by acting on the ventromedial hypothalamus [256, 490, 491], and was shown to be a trigger for rat copulatory behaviour [492]. Progranulin induced the proliferation of embryonic fibroblasts (R- cells) obtained from mice that lack functional insulin like growth factor-1 receptor (IGF-1). Progranulin was the only growth factor capable of inducing the proliferation of R- cells in the absence of IGF-1 and platelet-derived growth factor [494], through the activation of the p44/42 mitogen-activated protein kinase (MAP kinase) and the phosphatidylinositol 3-kinase (PI3 kinase) pathways and induction of cyclin D1 and cyclin B. Interestingly, these pathways are both involved in the signalling of IGF-1 and thus, may be the reason why progranulin can act in place of IGF-1 [280, 281]. In addition, progranulin participation in inflammatory responses has been demonstrated, because the molecule induced cellular migration during wound healing [261, 274]. Although the multi-functional nature of the progranulin was well characterized, a receptor for progranulin has yet to be identified.

In mammals, progranulin can not only exert its biological effects as an intact protein, but also a plethora of other functions as a result of proteolytic cleavage and production of functional smaller granulin peptides. For example, Epithelin 1/Granulin A (Epi1/GrnA) has been shown to induce proliferation of murine keratinocytes, as well as rat kidney cells NRK-SA6 in the presence of transforming growth factor β. However, Epi1/GrnA has also been shown to inhibit the DNA synthesis, and thus proliferation, of the epidermal cell line A431 and the human colon carcinoma cell line HCT116 [263, 297]. Interestingly, Epithelin 2/Granulin B (Epi2/GrnB) has been shown to antagonize the proliferative effects of Epi1/GrnA, as well as has some growth inhibitory effects of its own on A431 cells, albeit not to the same extent as Epi1/GrnA [263]. Therefore, it is possible for a small peptide such as goldfish granulin to have a range of biological effects, the majority of which remain to be characterized.

The structure, distribution and function of goldfish granulin set it apart from known mammalian granulins. Its association with the hematopoietic organs of the goldfish and its up regulation in cells that are undergoing proliferation suggests that it may be an important growth factor during hematopoiesis in goldfish. Its up regulation as a result of phagocyte activation, and high levels of expression in monocytes also suggest that the goldfish granulin characterized in this thesis, like mammalian granulin, may be involved in inflammatory and wound repair events. Studies on mammalian Epi1/GrnA and Epi2/GrnB have shown that these small peptides are remarkably stable [297] and that progranulin induced localized proliferation of cells and accelerated wound healing [252, 261, 274]. Whether goldfish granulin can modulate the inflammatory and would healing events is currently under investigation in our laboratory.

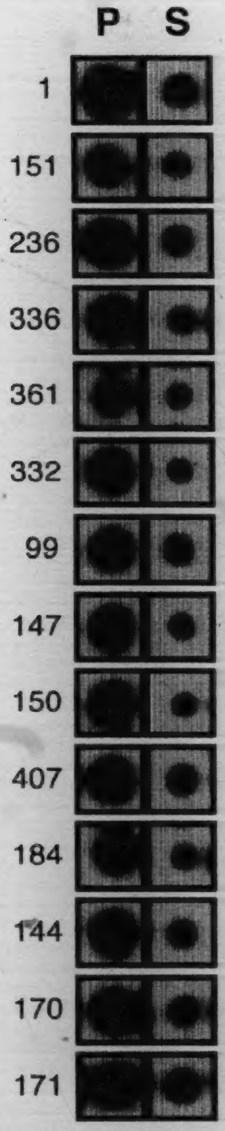

Figure 5.1 Goldfish granulin expression is associated with proliferating primary kidney macrophage cultures. Macroarray analysis of the differential expression of fourteen distinct granulin transcripts during proliferative (P) and senescence (S) phases of cultured primary macrophages of the goldfish. Numbers indicate different granulin transcripts whose expression was consistently upregulated during the proliferative phase of macrophages.

```
                                                                           M   V
  1 accgggctgcaggaattcggcacgagcagcagcaaaacaatctacaccagaggagacccagacagcaag atg gtt

     P   V   L   M   L   L   M   A   A   L   V   A   A   D   E   P   M   M   D   L
 76 cca gtg ttg atg tta ctc atg gca gct ctt gta gct gca gat gag cca atg atg gat ctc

     S   G   P   L   E   S   D   S   A   S   V   S   I   I   F   C   D   A   S   T
136 tca ggc cca tta gag tct gac agt gcc tct gtt tct atc ata ttc tgt gat gct tct act

     T   C   P   S   G   T   T   C   C   R   S   P   F   G   I   W   Y   C   C   P
196 aca tgt cct agc gga aca acg tgc tgt cgt tct cct ttt ggt att tgg tac tgc tgc cca

     F   S   M   G   Q   C   C   R   D   G   R   H   C   C   R   H   G   Y   H   C
256 ttc tca atg ggt cag tgc tgc aga gat gga cgc cat tgc tgt cgt cat ggt tat cac tgc

     D   A   S   S   T   L   C   L   R   G   W   L   K   L   P   S   S   A   E   P
316 gat gcg tca tcg acc ctt tgt ttg agg ggg tgg ttg aaa ctg cca tct tct gct gag ccg

     A   T   K   A   I   Q   K   P   Q   S   V   P   I   D   Q   A   L   K   W   K
376 gcc acc aag gct atc cag aaa cct cag tct gtg ccc att gac cag gct ctt aaa tgg aag

     S   E   T   E   S   V   H   C   D   G   N   L   Y   C   S   T   E   Q   F   C
436 agc gag act gag tca gtt cat tgt gat gga aat ctc tac tgc tca act gag cag ttc tgt

     C   K   T   A   A   G   Q   W   G   C   C   N   E   M   V   L   *
496 tgc aag aca gca gcc ggc cag tgg ggt tgc tgc aat gag atg gtg ttg taa gtaaacaacccc

559 cgtgacttgggtgcaggtcttattaaagacttaccaagtgcaagaggcttttgtcacactttcacgcagcattttccctt

639 tgttaatttgttgttatggacactcattagtgtcgactgtatactatcaatcatttattatcaccccatttggtcatgta

719 tttaaattgaactgtttaacttcttggttgtcaggtcttgtctctacttgctactattctgtgttgcatttactctgttt

799 ggattattaaaaactgcctgaattatcatgtgcatttattctaaccaagtgtgacagctttcagaagacaattactgttg

879 atatagattcaagctgaattaatgcaaaataaaactttaaagaactgcaaaaaaaaaaaaaaaaaa
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Figure 5.2 cDNA sequence of goldfish granulin with the predicted amino acid translation of the open reading frame. The cysteine residues composing the one and one half granulin motif are underlined.

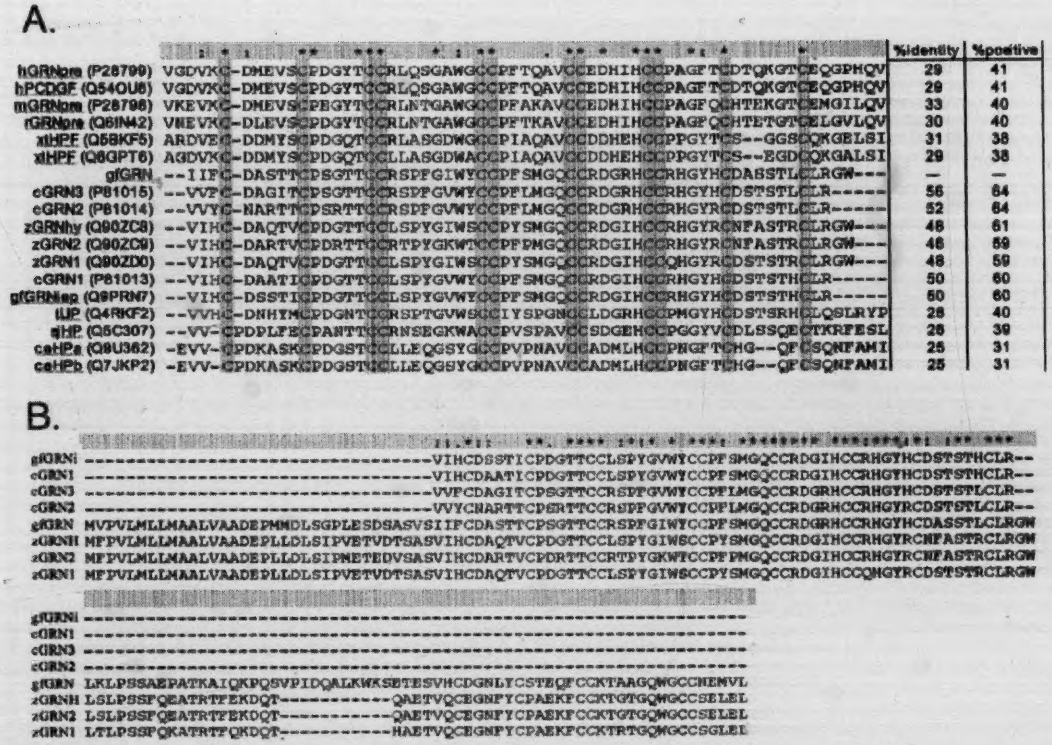
A.
%identity
%positive
hGRNpre (P28799)
hPCDGF (Q540U6)
mGRNpre (P28798)
rGRNpre (Q6IN42)
xlHPF (Q58KF5)
xlHPF (Q6GPT6)
gfGRN
cGRN3 (P81015)
cGRN2 (P81014)
zGRNhy (Q90ZC8)
zGRN2 (Q90ZC9)
zGRN1 (Q90ZD0)
cGRN1 (P81013)
gfGRNiep (Q9PRN7)
ILIP (Q4RKF2)
alHP (Q5C307)
ceHPa (Q9U362)
ceHPb (Q7JKP2)
B.
gfGRNi
cGRN1
cGRN3
cGRN2
gfGRN
zGRNH
zGRN2
zGRN1

Figure 5.3 Goldfish granulin sequence analysis.(A) Amino acid alignment of the known granulin sequences of fish. zGRNH – zebrafish hybrid granulin (AAK58710), zGRN2 – zebrafish granulin 2 (NP997921), zGRN1 – zebrafish granulin 1 (AAK58708), gfGRNi – goldfish granulin identified from intestine (AAB47075), cGRN1 – carp granulin 1 (AAB26496), gfGRN – goldfish granulin, cGRN3 – carp granulin 3 (AAB26498), cGRN2 – carp granulin 2 (AAB26497).(B) Amino acid alignment of the conserved cysteine area of *Xenopus* (Xgrn (AAY26493)), human (hGRN (AAH10577)), zebrafish (zGRNH, zGRN1, zGRN2), goldfish intestinal granulin peptide (gfGRNi), carp (cGRN1, cGRN2, cGRN3), *C. elegans* (C.elGRNa (CAB54304), C.elGRNb (CAB54305)), and goldfish granulin (gfGRN).

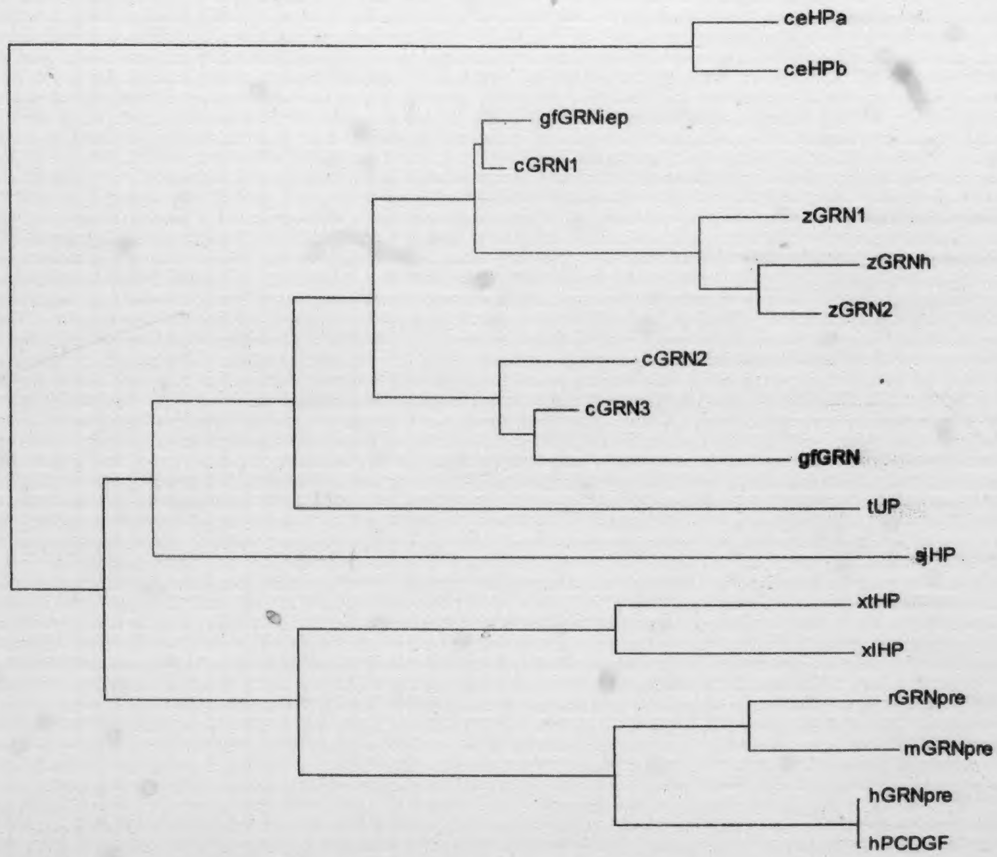
ceHPa
ceHPb
gfGRNiep
cGRN1
zGRN1
zGRNh
zGRN2
cGRN2
cGRN3
gfGRN
tUP
sjHP
xtHP
xlHP
rGRNpre
mGRNpre
hGRNpre
hPCDGF

Figure 5.4 Phylogenetic tree of selected granulins. Goldfish granulin groups closely to carp granulins-3 and granulin-2, which are closely associated with the zebrafish granulin-1, granulin-2 and zebrafish hybrid granulin, as well as carp granulin-1 and goldfish granulin identified from intestinal exudates. All of the progranulin peptides from *Xenopus*, human, mouse and rat group closely and all are out grouped by a granulin-like peptides identified in *C. elegans*. The tree was bootstrapped 10,000 times to ensure accuracy. Abbreviations are the same as in Figs. 5.3.

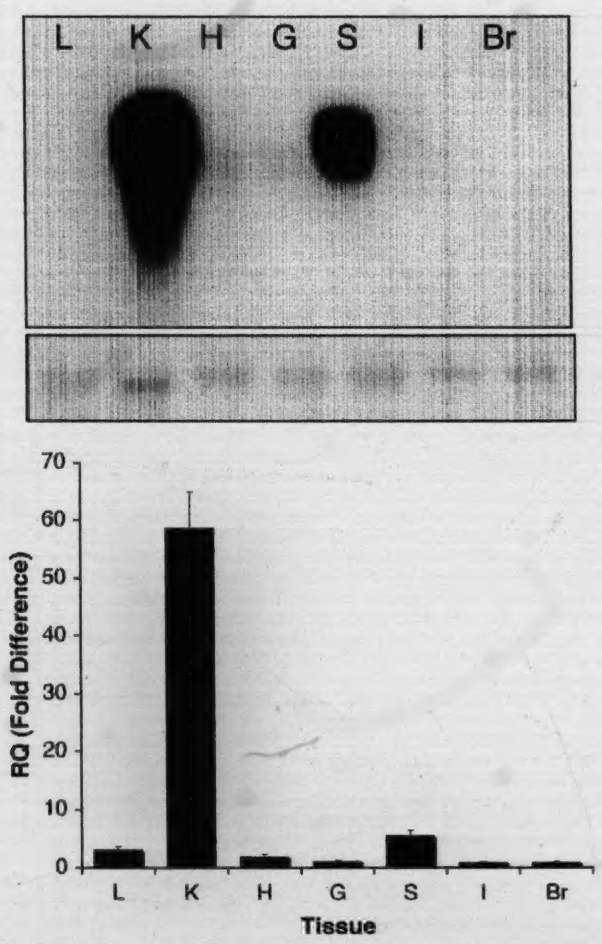

Figure 5.5 Granulin mRNA expression analysis in tissues of goldfish. Northern blot (A) of goldfish granulin transcript expression in various tissues (L=liver, K=kidney, H=heart, G=gill, S=spleen, I=intestine, Br=brain), Kidney and spleen show the highest level of transcript expression. 18S ribosomal RNA was used as a loading control. (B) Real-time PCR analysis of granulin expression in different tissues of the goldfish. The data are from five independent experiments (n = 5).

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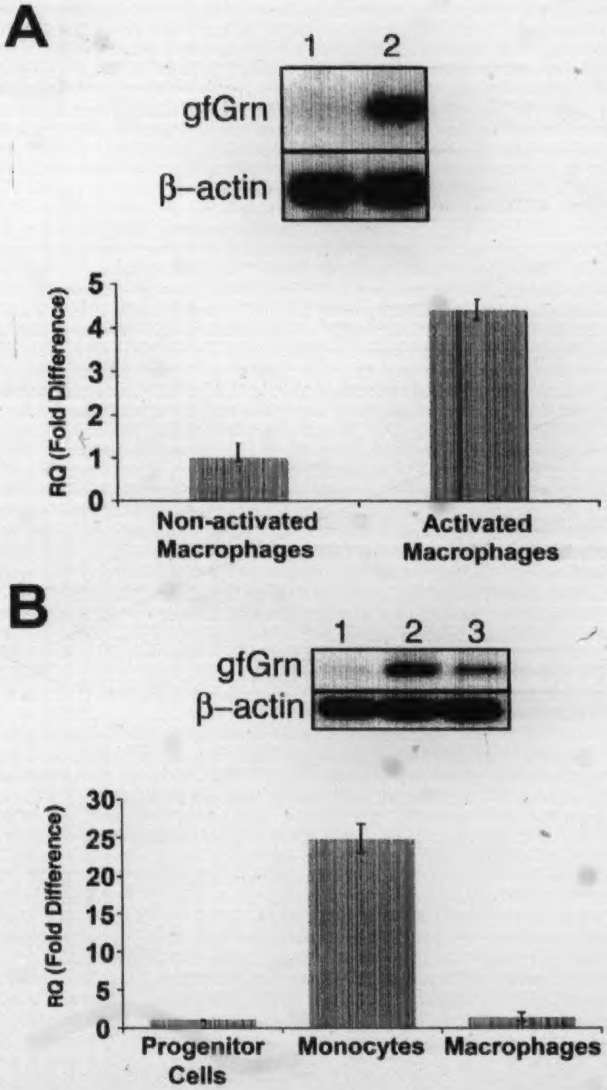

Figure 5.6 Goldfish granulin mRNA expression analysis in sorted and activated primary kidney macrophages. Quantitative PCR and RT-PCR analysis of granulin transcript expression in goldfish macrophages. A1 and A2 represent granulin expression in non-activated and activated goldfish macrophages, respectively. B1, B2 and B3 represent granulin expression in progenitor cells, monocytes and macrophages, respectively. β-actin was used as a loading control in RT-PCR analyses. The data for real time PCR are from five independent experiments. RT-PCR data are from a representative experiment of five that were performed.

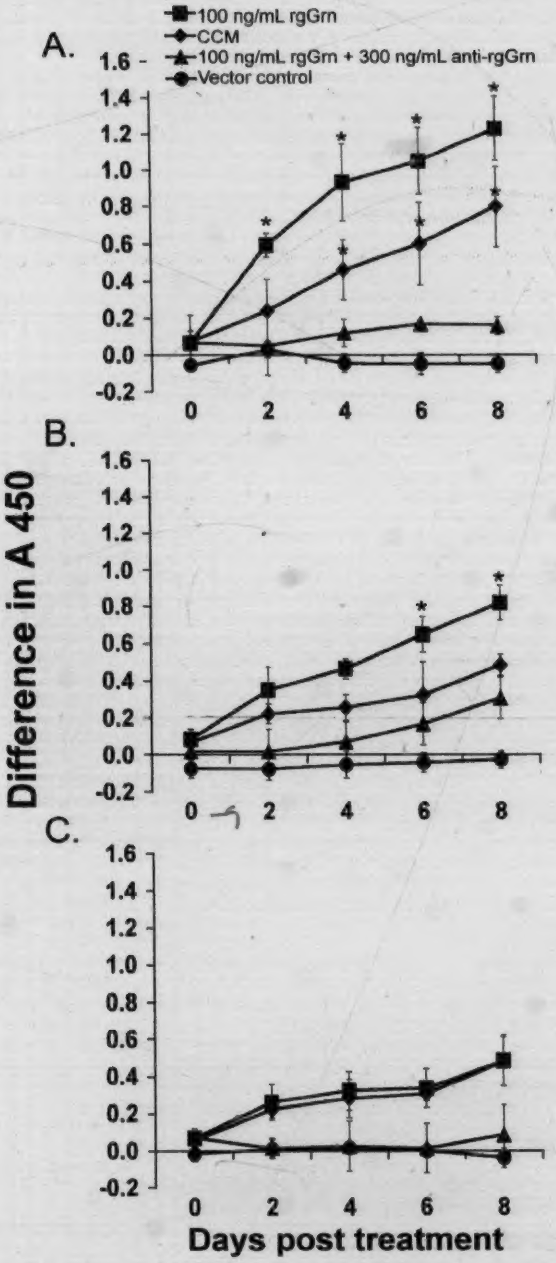

Figure 5.7 (A-C) The induction of macrophage proliferation by recombinant goldfish granulin (rgGrn). Sorted progenitor cells (A), monocytes (B) and macrophages (C) were treated with 100 ng/mL recombinant goldfish granulin or 100 ng/mL recombinant granulin and 300 ng/mL anti-goldfish granulin antibody and analyzed for their ability to proliferate using a BrdU assay. The proliferation OD values were normalized to cells alone controls. Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=8).

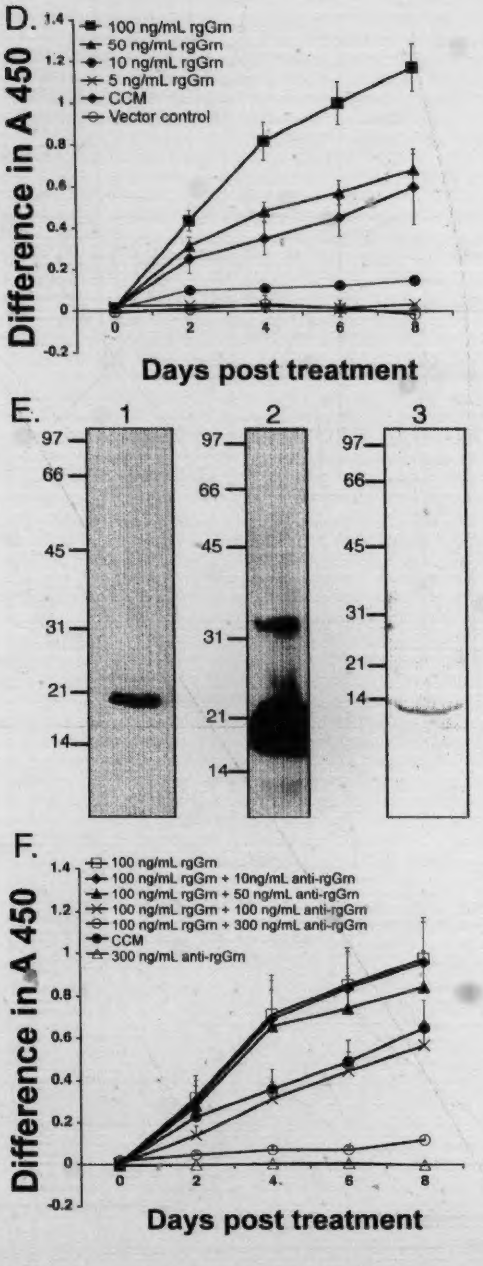

Figure 5.7 (D-F) Goldfish granulin is secreted by primary kidney macrophages and its effects are dose dependent. The proliferative response of goldfish macrophage progenitor cells after addition of recombinant goldfish granulin (rgGrn) in concentrations ranging between 5 ng/mL and 100 ng/mL, compared to CCM and vector controls (D); Western blot using the anti-HIS antibody to detect the recombinant goldfish granulin (1), the antibody generated against the recombinant to detect the recombinant in expression bacterial lysates (2) or the anti-granulin antibody to detect native goldfish granulin in PKM supernatants (3) (E); The proliferative response of goldfish macrophage progenitor cells exposed to 100 ng/mL of rgGrn mixed with different concentrations (10 ng/mL to 300 ng/mL) of anti-rgGrn antibody (F). Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) is denoted with (*). Each point on the graphs represents mean ± SEM (n=8).

Chapter 6

Characterization of the Leukemia Inhibitory Factor (= M17) and Leukemia Inhibitory Factor Receptor of the Goldfish[1]

6.1 Introduction

Cytokines of the interleukin-6 family, [IL-6, IL-11, oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF)], are functionally pleiotropic and participate in diverse biological processes including bone formation, neuronal survival and development, inflammation, activation of immune cells, hematopoiesis and hormone production. Each of these molecules has distinct functions; however, one of the hallmarks of the cytokines in this group is a large degree of redundancy shared between family members. This redundancy is in part due to the fact that each of these cytokines signal through a cell surface receptor complex containing the glycoprotein gp130 [298, 495].

There is a significant body of work that has addressed the functions of each of the IL-6 family cytokines in humans, mice and rats, and members of this family have been identified and partially characterized in chickens [496, 497]. The recent findings of an IL-6-like molecule in the Japanese puffer fish [407], a molecule resembling an IL-6 family cytokine in carp [403], and zebrafish, and the identification of a leukemia inhibitory factor receptor-like molecule in the goldfish [47], suggest that the IL-6 cytokine family may be of importance in host defence of lower vertebrates. At present, the functional roles of these cytokines in fish are largely unknown. Sera isolated from virally challenged carp and trout [498], as well as supernatants from mitogen-stimulated trout leukocytes [499], were shown to possess molecules that are recognized by the anti-IL-6 antibodies. Moreover, Atlantic salmon hepatocytes treated with recombinant human IL-6 exhibited an up regulation in the transcription of the salmon acute phase protein, serum amylois A (A-SAA) [500]. To date, no functional analyses were done in a homologous system (i.e., using fish cytokine and fish cells), tests that must be done in order to understand the contribution of these cytokines in host defence of fish.

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[1] A version of this chapter has been published:
Hanington and Belosevic. 2007. Interleukin-6 family cytokine M17 induces differentiation and nitric oxide response of goldfish (*Carassius auratus* L.) macrophages. Developmental and Comparative Immunology. 31: 817-29.
Hanington and Belosevic. 2005. Characterization of the leukemia inhibitory factor receptor in the goldfish (*Carassius auratus*). Fish and Shellfish Immunology. 18: 359-69.

In this chapter, I report on the identification of a goldfish IL-6 family cytokine that bears strong similarities to carp and zebrafish M17, and the characterization of goldfish M17 (LIF) in terms of its ability to induce the proliferation, differentiation and activation of goldfish macrophages.

6.2 Experimental Design

6.2.1 In silico analysis of goldfish M17 and LIFR

Using goldfish genomic DNA as a PCR template, the genomic organization of goldfish M17 was identified and compared to the genomic organizations of known mammalian IL-6 family cytokines. Furthermore, the sequence similarities between goldfish M17 and LIFR to known mammalian IL-6 family cytokines and IL-6 family cytokine receptors were assessed. These *in silico* assays confirmed that M17 was a member of the IL-6 cytokine family and that it was leukemia inhibitory factor (LIF).

Furthermore, goldfish LIFR resembled mammalian LIFR in its predicted amino acid primary structure.

6.2.2 *Expression analysis of goldfish LIF and LIFR*

To determine how goldfish LIF was related to mammalian IL-6 cytokines, the expression patterns of goldfish LIF and LIFR in relation to β-actin in the kidney, spleen, liver, heart, gill, intestine, brain, and blood leukocytes were assessed. In addition to analyzing tissue expression, LIF and LIFR expression in sorted goldfish progenitor cells, monocytes and macrophages were also determined.

6.2.3 *Recombinant expression of goldfish LIF*

Goldfish LIF was expressed using the pET SUMO and pET 151 TOPO prokaryotic expression vectors according to the manufacturer's protocols and the methods described in chapter 3. The identity of the recombinant proteins was confirmed using mass spectrometry and polyclonal antibodies were generated against the recombinant protein. The most effective recombinant protein was generated using the pET SUMO vector and was designed to incorporate the entire secreted goldfish LIF molecule, from the end of the signal peptide to stop codon of the transcript.

6.2.4 *Induction of primary kidney macrophage proliferation by recombinant goldfish LIF*

The effects of recombinant goldfish LIF (rgLIF) on PKM proliferation were assessed using the cell proliferation ELISA BrdU colorimetric assay (Roche). PKM, grown as described in chapter 3, were sorted using a FACS Calibur flow cytometer (Becton Dickinson) based on size and complexity. Cells were labelled with BrdU labelling reagent as described in chapter 3, and after labelling, progenitor cells, monocytes and macrophages were washed and seeded in 96-well plates in 50 µL of incomplete culture medium and treated with 50 µL of 100 ng/mL, 20 ng/mL, 10 ng/mL, 1 ng/mL and 0.1 ng/mL of rgLIF mixed with either incomplete cell culture medium or complete medium. Control treatments included incomplete medium alone, CCM and an expression vector control. Measurements were taken every two days after day 0 of treatment in triplicate using PKM established from 8 individual fish (n=8).

6.2.5 Induction of monocyte differentiation by recombinant goldfish LIF

Using the same treatments as those in the proliferation experiment, the ability of rgLIF to influence the development of goldfish monocytes/macrophages was assessed using flow cytometry. Sorted cells from PKM cultures established from 5 individual fish (n=5) were treated in 500 µL volumes and analyzed every two days after day 0 of treatment based on their size and internal complexity. Gate outlines employed in previous experiments were used as guides to determine differences between the treatment groups and controls.

6.3 Results

6.3.1 Characterization of goldfish Leukemia Inhibitory Factor (LIF)

6.3.1.1 Sequence analysis and comparison

Goldfish LIF (DQ961993) is a 672 bp transcript that encodes for a predicted peptide of 224 amino acids. It has a predicted N-terminal signal peptide that is cleaved between amino acids 40 and 41, resulting in a putative active peptide of 182 amino acids. The predicted LIF peptide has 90% amino acid identity with carp (*Cyprinus carpio*) M17 (AY102633), and 35% identity with a putative M17 identified in the puffer fish (*Tetraodon nigriviridis*) (CAF99247). Goldfish LIF has 73% identity with both of the recently identified zebrafish (*Danio rerio*) M17s (XM679703 and XM678310) which only differ by 2 amino acids (Fig. 6.1). BLAST analysis suggests that the M17s are most closely related to chicken (*Gallus gallus*) CNTF; however, *in silico* analysis of all M17s suggests that they possess a signal peptide for secretion from cells, whereas CNTFs of chickens and mammals do not. Both the carp [403] and zebrafish M17 genes also possess a similar genomic arrangement although the zebrafish M17 genes appear to have a large number of extra amino acids on their N terminal end that are not present in goldfish LIF or carp M17 transcripts.

6.3.1.2 Quantitative transcript expression analysis

Quantitative PCR analysis of goldfish LIF using the spleen as a reference tissue suggested that goldfish LIF expression was highest in the brain, a 22 times higher expression when compared to that in the spleen. Expression levels were also higher in the kidney (9 times higher) and isolated leukocytes (5 times higher) than the levels observed in the spleen. The spleen, liver, heart, gill and intestine all displayed low expression levels (Fig. 6.2A). Goldfish LIF was expressed in progenitor cells, monocytes and macrophages, sorted using flow cytometry. The expression was 2.5 times higher in the macrophages and 1.5 times higher than that seen in the progenitor cells which were used as a reference tissue (Fig. 6.2B). Goldfish LIF expression increased 8.5 times when macrophages were treated with LPS and MAF (Fig. 6.2C).

6.3.1.3 Analysis of recombinant goldfish LIF

The purity of the goldfish rgLIF sample was assessed by SDS-PAGE stained with coomassie G250. The molecular weight of the recombinant goldfish LIF with the SUMO fragment and 6x HIS tag was 30 kDa, and appeared to purify as a doublet. Goldfish rgLIF was isolated from bacterial lysates and the elution fractions were again affinity purified (Fig. 6.3). No co-purifying proteins were seen after the second affinity purification and the identity of the sample was confirmed using Western blot analysis and mass spectrometry. Both bands of the doublet were found to be rgLIF.

6.3.1.4 Nitric oxide response of primary kidney macrophages induced by recombinant LIF

When macrophage cultures were treated with 15 μg/mL or greater of rgLIF they exhibited a nitric oxide response (~4 μM nitrite) measured by nitrite concentration using the Griess reaction. This effect was enhanced to ~8 μM nitrite when macrophages were treated with rgLIF and 2 μg/mL LPS. The empty vector control did not induce nitric oxide production when added to goldfish macrophages either alone (~1 μM nitrite) or in conjunction with 2 μg/mL LPS (~2 μM nitrite), and the largest response was seen in the positive control consisting of MAF and 2 μg/mL LPS (~10 μM nitrite) (Fig. 6.4).

6.3.1.5 Enhancement of the proliferation of primary goldfish macrophages in the presence of recombinant goldfish LIF

Goldfish rgLIF was used at concentrations of 100 ng/mL, 20 ng/mL, 10 ng/mL, 1 ng/mL, and 0.1 ng/mL in a BrdU proliferation assays using sorted goldfish macrophage progenitor cells, monocytes or macrophages. At all concentrations of rgLIF, for each sorted population of PKM cells, no significant increase in proliferation was observed after treatment with rgLIF alone (data not shown). However, when rgLIF was added at concentrations of 10 ng/mL or higher in conjunction with CCM, a significant increase in proliferation over CCM alone was observed in the progenitor cell population (Fig. 6.5 A). This increase was not seen in the monocyte or macrophage populations (Fig. 6.5 B and Fig. 6.5 C). There was no change in the proliferation of any of the sorted populations in the presence of the empty vector and the LPS control groups.

6.3.1.6 Differentiation of goldfish monocytes induced by recombinant goldfish LIF

Goldfish rgLIF at concentrations of 100 ng/mL, 20 ng/mL, 10 ng/mL, 1 ng/mL and 0.1 ng/mL was added to sorted goldfish progenitor cell, monocyte or macrophage cultures. The cultures were monitored every 24 hours for 8 days by flow cytometry to assess the effect of rgLIF on differentiation of each sorted PKM subpopulations. Concentrations of 10 ng/mL of rgLIF and higher had no effect on the differentiation of sorted goldfish progenitor cells or macrophages (Fig. 6.6 A and B). However the same concentrations of rgLIF induced pronounced differentiation of sorted monocytes (Fig. 6.6 C).

6.3.2 Characterization of goldfish Leukemia Inhibitory Factor Receptor (LIFR)

6.3.2.1 Sequence analysis of goldfish LIFR

The putative goldfish LIFR was found to have an ORF of 2481 nucleotides, which translated into 827 amino acids (GenBank accession number AY749167). The cytoplasmic region had the box-1 motif 'FYPXIPXPX' characteristic for LIFR. The 3 prolines within this motif were critical for Jak-1 and Jak-2 association and activation. The box-2 motif was still present, but did not share the same high cross-species conservation as box-1. The extracellular portion retained the second cytokine binding motif 'WSXWS' but the first motif, although structurally present, did not share sequence homology with human or chicken LIFR.

Four fibronectin type 3 domains were identified in the sequence of goldfish LIFR and analyzed for conserved protein motifs. These domains aided in classifying the goldfish protein as a member of the IL-6 family of cytokine receptors. These domains coincide with the chicken and human LIFRs, although the size and position of each domain was different (Fig. 6.7).

BLASTX analysis identified rat, chicken, human and mouse LIFR as the four proteins with the highest amino acid homologies to the putative goldfish LIFR, respectively. The percent amino acid identity with the chicken, rat, mouse and human LIFR sequences was 24%, 26% 26% and 26%, respectively.

The goldfish putative LIFR is 269 amino acids shorter than the human LIFR, which is the longest LIFR sequence we analysed (i.e. 1097 amino acids). The region of amino acids absent in the goldfish putative LIFR was primarily located in the cytoplasmic portion of the protein. The putative goldfish LIFR had a predicted transmembrane region between amino acids 702 and 724, and a cytoplasmic region of 103 amino acids. In contrast, human LIFR had a predicted transmembrane region between amino acids 833 and 858, and a cytoplasmic region of 239 amino acids.

6.2.2.2 The expression of LIFR in goldfish tissues and activated macrophages

RT-PCR analysis was performed to determine relative tissue expression mRNA of the LIFR. Total RNAs were isolated from kidney, spleen, liver, heart, gill, brain, muscle and testes, as well as primary goldfish kidney progenitor cells, monocytes and macrophages. LIFR expression was detected in the gill, kidney and brain. No LIFR message was observed in the spleen, heart, liver, muscle or testes. Macrophages exhibited higher mRNA LIFR expression compared to that in monocytes and progenitor cells (Fig. 6.8).

6.5 Discussion

This chapter details the identification and characterization of an IL-6 like cytokine from the goldfish. This cytokine, initially named M17 after its original identification in carp [403], has subsequently been found in zebrafish and shares the highest sequence identity with the presently known sequences of mammalian and avian ciliary neurotrophic factors. The genomic analysis of the putative goldfish M17 justified the renaming of the goldfish transcript to leukemia inhibitory factor (LIF).

Quantitative PCR analysis of goldfish LIF expression indicated that it was highly expressed in the brain and kidney, mimicking the expression exhibited by carp M17 [403]. Moreover, goldfish LIF was upregulated in activated goldfish macrophages, and was found to be differentially expressed in sorted goldfish macrophage subpopulations where macrophages had higher LIF expression compared to monocytes and progenitor cells. The treatment of monocyte subpopulation with rgLIF induced rapid differentiation of monocytes to macrophages indicating functional importance for this molecule in myelopoiesis.

Goldfish LIF had highest sequence identity with chicken CNTF. Like CNTF [501], goldfish LIF was highly expressed in the brain and lower expression in other tissues tested. Phylogenetic analysis groups goldfish M17 with mammalian CNTF, CT-1 and LIF. Detailed analyses of the M17 mRNA of carp [403], goldfish M17 (this study) and zebrafish, indicated the presence of a signal peptide that would direct the M17 peptide extracellularly. Both LIF and CT-1 possess signal peptides of a similar nature, however, CNTF does not have a signal peptide that would perform a similar function. Likewise, the promoter regions and genomic sequence of zebrafish and carp M17 are very similar to the promoter region of the mammalian LIF. In fact, chromosomal analysis of zebrafish M17 suggests that it shares a syntenic relationship with human and murine LIF and OSM. The synteny shared between the zebrafish M17 and human and mouse LIF and OSM suggest that M17 could possibly be an ancestral molecule to LIF and OSM.

LIF and CNTF are multifunctional cytokines that exert their biological effects by binding to a heterodimeric receptor composed of a low affinity LIF receptor and the gp130 receptor subunits. Much of the functional redundancy observed within the IL-6 cytokine family is due to the presence of the gp130 subunit in all of the receptor formats for IL-6 family cytokines [298].

LIF has diverse functions, including being required for blastocyst implantation [502-505] and the normal development of hippocampal [506] and olfactory receptor neurons [507]. LIF prevented the differentiation of pluripotent stem cells [318, 508-511] and it has been implicated in various aspects of bone formation, adipocyte lipid transport [512], neuronal formation and survival [513], platelet formation and acute phase protein production [514]. Importantly, LIF has also been implicated in affecting proliferation and differentiation during myelopoiesis [515-518].

Like LIF, CNTF has very diverse functions. The role CNTF plays in the regulation of neuronal development appears to be greater than that of LIF [519-522]. LIF and CNTF appear to have overlapping functions. For example, knockout mice for either LIF or CNTF, having same genetic background, showed no abnormalities of motor function (LIF$^{-/-}$) or just a slight alteration of it (CNTF$^{-/-}$); nevertheless, significant functional motor deficits were found in the double knockouts (CNTF$^{-/-}$, LIF$^{-/-}$) [523]. CNTF was also shown to induce of the production of acute phase proteins by hepatocytes [524]. Unlike LIF, CNTF does not have a secretory signal and is located in the cytosol of healthy cells [522].

Functionally, goldfish LIF resembles mammalian LIF in its ability to influence myelopoiesis. The rgLIF used for functional studies presented in this thesis appeared as a doublet when run on SDS-PAGE. Both bands of the doublet were analyzed by mass spectrometry and were identified as LIF. rgLIF induced a nitric oxide response in goldfish macrophages alone and in combination with LPS or with MAF. Of the three cytokines that share highest similarities with teleost M17 (LIF, CT-1 and CNTF), LIF is the most potent inflammatory mediator being implicated in arthritis and other inflammatory conditions in humans [298, 495]. LIF has also been shown to induce the differentiation of murine M1 myeloid leukemia cells [313, 525, 526], similar to that induced by rgLIF in goldfish monocytes (Fig. 6-6).

The ability of rgLIF to enhance goldfish macrophage progenitor cell proliferation in combination with CCM, suggests that rgLIF has additional mammalian LIF-like properties [315]. The possibility that goldfish macrophage CCM may contain some secondary growth factors that are required for the expression of rgLIF growth-promoting properties, like those described for mammalian LIF [315], is currently under investigation in our laboratory.

The treatment of progenitor cells with rgLIF did not appear to arrest the differentiation of this sorted macrophage cell subpopulation. Mammalian LIF has been commonly used to prevent the differentiation of mammalian pluripotent stem cells [318, 508-511] into their committed progeny. Therefore, this would suggest that goldfish sorted macrophage progenitor subpopulation has been already committed towards the myeloid differentiation pathway. My results suggest that goldfish LIF shares sequence similarity and the expression patterns of CNTF, but that it possesses LIF-like functions, suggesting that goldfish LIF (=M17) may be ancestral to the divergence of CNTF and LIF in higher vertebrates.

Goldfish LIFR contained all conserved mammalian LIFR domains, suggesting a similarity in function. Several important functional domains identified in mammalian LIFR were also present in goldfish LIFR. For example, the box-1 region has been shown to participate in the control of hematopoietic events induced by LIF binding to LIFR [6]. The box 1 region of the LIFR has been shown to have a significant role in Jak-1 and Jak-2 association and activation of STAT3 [527, 528], which controls the differentiation and growth arrest of myeloid leukemic cells [350]. The box-1 region, which is conserved across known LIFR molecules, is present in the predicted amino acid sequence of goldfish LIFR. On the other hand, the box-2 motif of goldfish LIFR shared less homology with the mammalian LIFRs, and although the box-2 motif has been shown to be responsible for Jak-1 association, this association is not required for the LIFR involvement in hematopoietic events [527].

Goldfish LIFR also has the two cytokine-binding motifs (WSXWS) [529, 530]. There was a slight difference in the C-terminal cytokine-binding motifs (CBM), where the first serine was substituted by glycine. This difference may be significant enough to alter the interaction between the LIFR and gp130, which may be assessed only after the gp130 of the goldfish, has been cloned and sequenced. The N terminal CBM was associated with the Ig-like domain of the receptor. This CBM has been shown to be responsible for ligand specificity [366, 531-535]. In the goldfish, this CBM retained the conserved WSXWS motif, as well as a majority of the conserved regions responsible for ligand contact in the Ig-like domain. The fibronectin III domains were also found to be highly conserved. These regions are important for formation of signalling receptor complexes, but the underlying mechanisms of this process have not been characterized [536].

The expression of LIFR was observed in the gill, kidney and brain. The high expression of the LIFR in the gill might suggest that LIF/LIFR interaction in the gill may be required for the development of appropriate host defence mechanisms at mucosal surfaces. The expression of LIFR in the brain was expected since LIFR has been shown to be involved in the response of the CNS and PNS to injury and infection [537]. LIF may not only influence the inflammatory response by regulating the function of the immune cells by binding to LIFR, but also indirectly by inducing the secretion of secondary inflammatory mediators, such as neuropeptides [538]. LIF production has been shown to be required for neuronal death and neurogenesis following axotomy of olfactory neurons [309]. LIF and LIFR expression has also been associated with the differentiation of astrocytes [539] and the maintenance of adult neural stem cells [540]. LIFR heterozygote mice showed a reduced number of adult neural stem cells in comparison to homozygote LIFR mice, suggesting that LIFR/gp130-mediated signalling likely inhibits the restriction of stem cells to a glial cell fate, thereby maintaining the neural stem cell population [539].

Further studies are needed to fully understand the role of teleost IL-6 family of cytokines, including goldfish LIF. With the cloning of a LIF receptor in the goldfish [47] studies can be done to assess the ability of rgLIF to interact with this receptor. Furthermore, current attempts to characterize goldfish macrophage progenitor cells in our laboratory [46] will aid in determining whether rgLIF influences progenitor cell behaviour in teleosts.

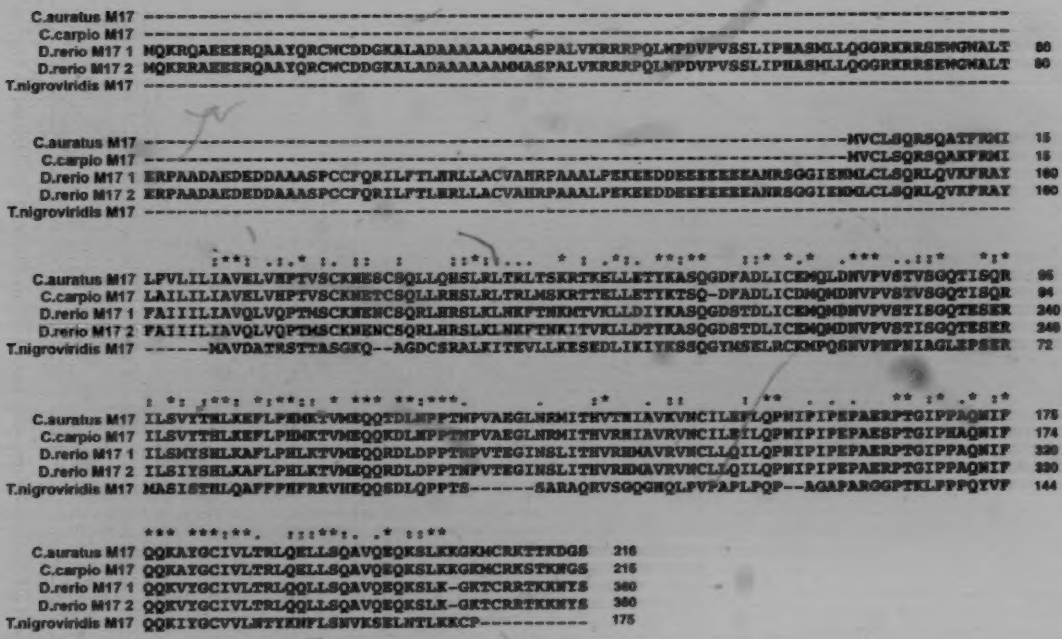
C.auratus M17
C.carpio M17
D.rerio M17 1
D.rerio M17 2
T.nigroviridis M17

Figure 6.1 Amino acid alignment of fish M17/LIF like peptides. Amino acid alignment of goldfish (*Carassius auratus*) M17 (= goldfish LIF), carp (*Cyprinus carpio*) M17, zebrafish (*Danio rerio*) M17-1 and M17-2 and a M17-like transcript from the pufferfish (*Tetraodon nigroviridis*). Amino acids conserved in all sequences are indicated with a star (*), amino acids that have strong conservation are indicated with (:) and weak conservation is indicated with (.).

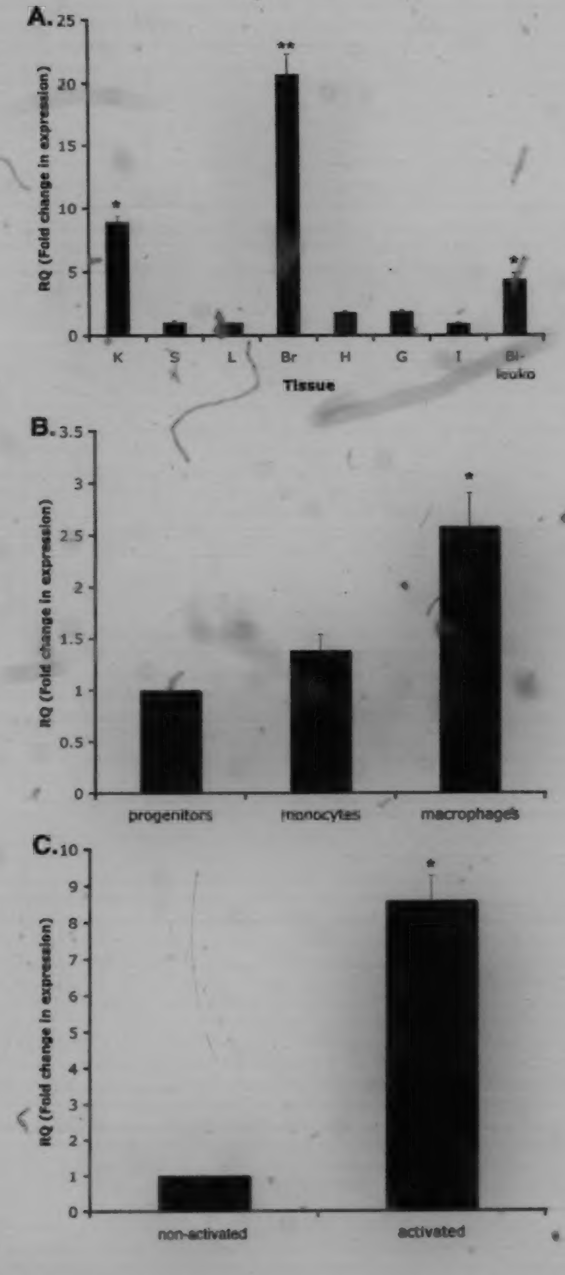

Figure 6.2 Quantitative PCR of goldfish LIF expression in different goldfish tissues. Goldfish LIF exhibited the highest expression levels in the brain (Br), kidney (K), and peripheral blood leukocytes (Bl-leuko). No significant expression was detected in the spleen (S), liver (L), heart (H), gill (G) or intestine (I) (A). Goldfish LIF was differentially expressed in goldfish macrophage progenitor cells, monocytes and macrophages with the highest expression levels seen in sorted macrophages (n=5) (B). LIF expression was induced by activating goldfish macrophages with LPS and macrophage activation factor, MAF (n=5) (C). Data were normalized to spleen (A), progenitor cells (B) and non-activated macrophages (C), and compared to the expression of β-actin. Statistical significance was determined using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) compared to the reference tissue is indicated by (*) (n=5).

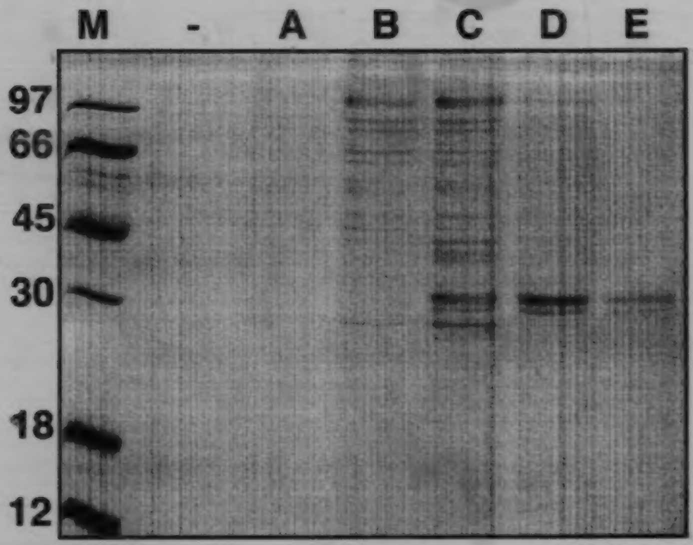

Figure 6.3 SDS-PAGE of purified recombinant goldfish leukemia inhibitory factor (LIF). Coomassie G250 stained SDS-PAGE showing the purity of the recombinant goldfish LIF. M—size marker,- blank well, A – non-induced bacteria not transformed with goldfish LIF construct, B - lysates of non-induced bacteria not transformed with the goldfish LIF construct, C - lysates of bacteria induced and transformed with goldfish LIF, D - first purification of recombinant goldfish LIF, E - second purification of goldfish LIF.

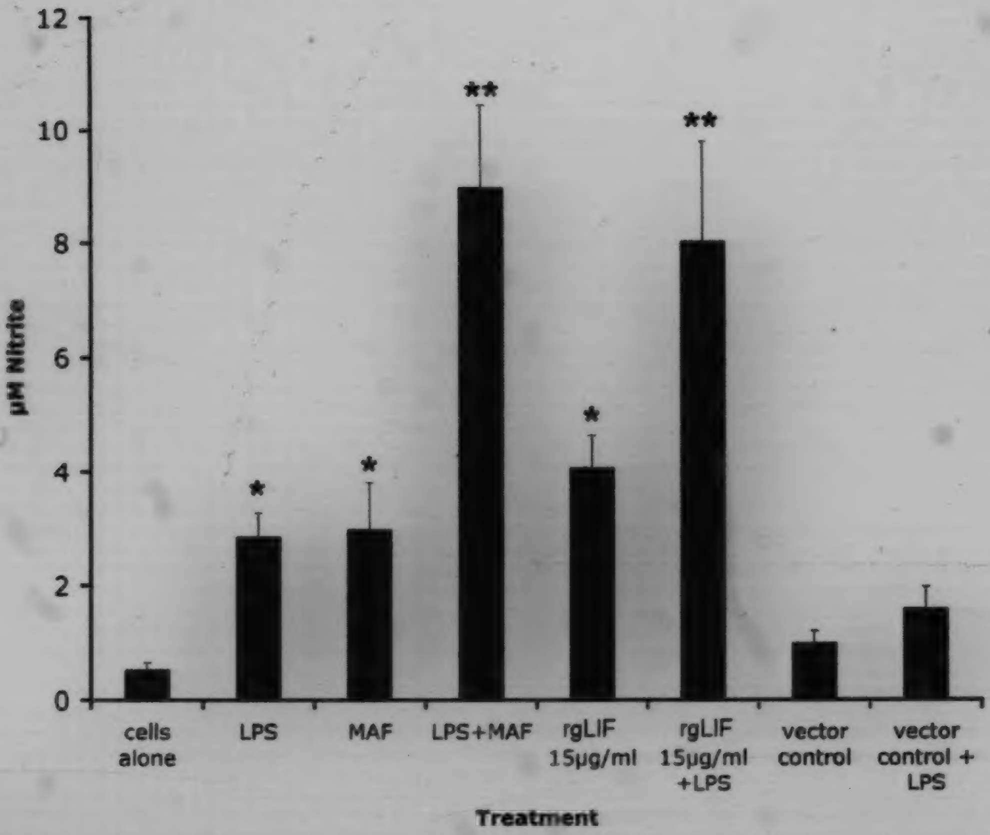

Figure 6.4 rgLIF induces nitric oxide response of goldfish primary kidney macrophages. Nitrite production by goldfish macrophages after treatment with 15 μg/ml of recombinant rgLIF. Fifteen μg/ml of rgLIF induced nitric oxide response in goldfish macrophages, which was enhanced further after treatment of cells with rgLIF and 2 μg/mL LPS. Empty vector with or without 2 μg/mL LPS did not induce significant nitrite production. This is a representative experiment of 8 independent experiments that were done using cell cultures established from individual fish. Statistical significance calculated using one-way ANOVA and Tukey post hoc analysis, ($P < 0.05$) compared to the cells alone control is indicated by (*) (n=4).

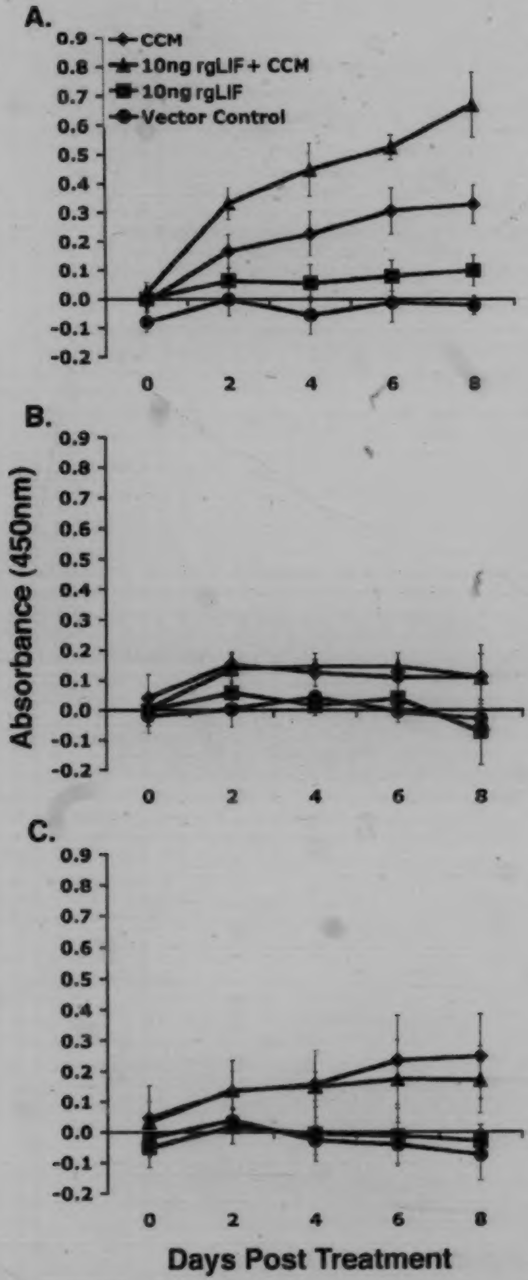

Figure 6.5 rgLIF enhances CCM-induced proliferation of sorted progenitor cells. Ten ng/mL of goldfish rgLIF induced proliferation of sorted goldfish macrophage progenitor cells in conjunction with macrophage cell conditioned medium, CCM (A). This proliferative effect was not observed after addition of rgLIF to sorted monocytes (B) or macrophages (C). Proliferation values were normalized by subtracting values obtained for non-treated cells and compared to cells treated with CCM alone or empty vector controls (A, B and C). This is a representative experiment of 8 that were done using cell cultures established from 8 individual fish.

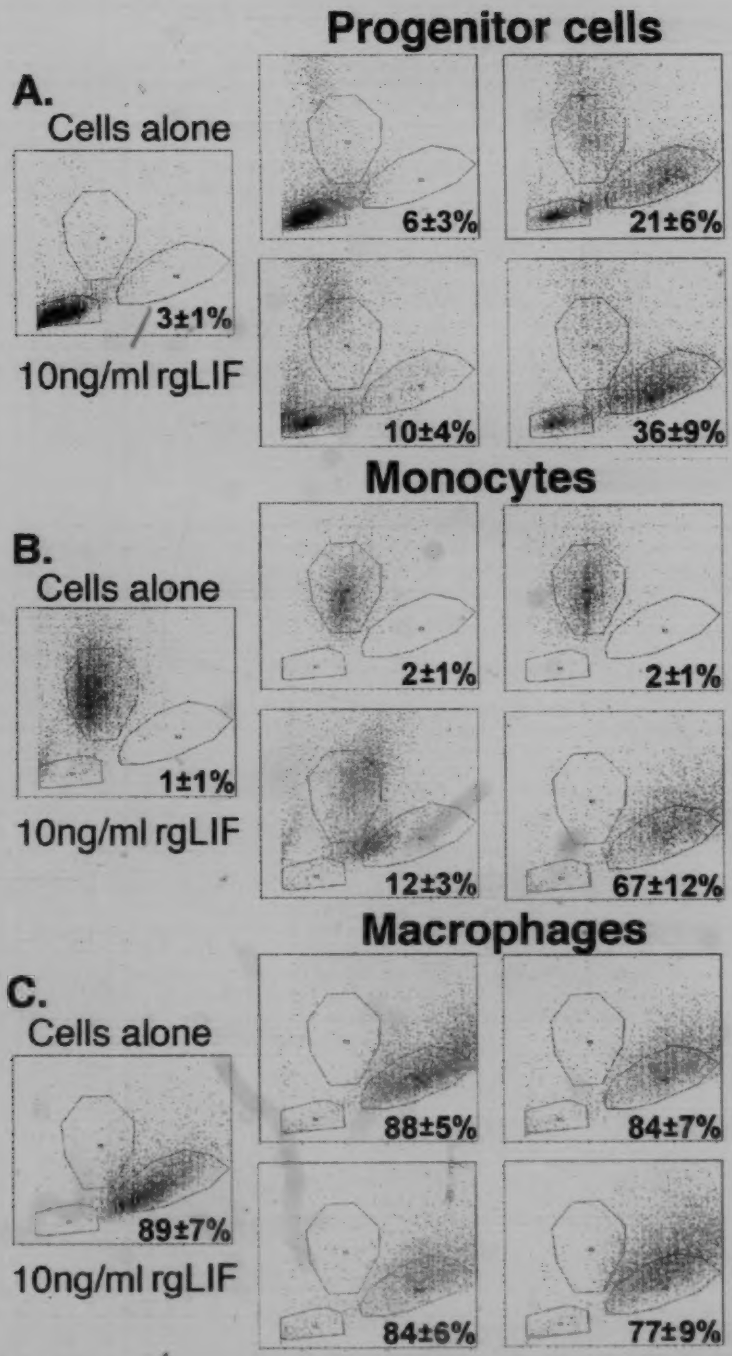

Figure 6.6 rgLIF induces monocyte differentiation into macrophages. Recombinant goldfish M17 induced differentiation of sorted goldfish monocytes into macrophages (B), but not macrophage progenitor cells (A) or sorted goldfish mature macrophages (C). This is a representative experiment of 8 that were done using cells cultures established from individual fish. Numbers represent the average percentage of events that occur in the macrophage gate plus/minus the standard error.

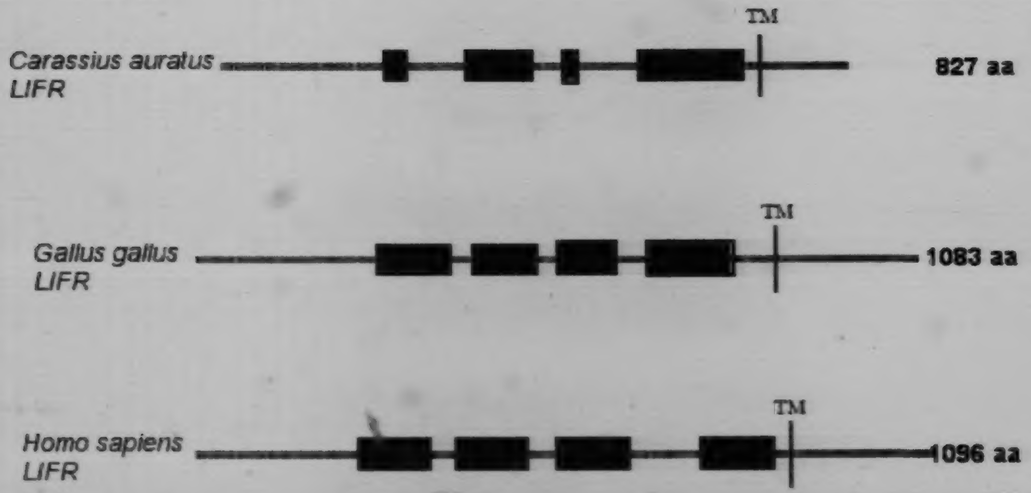
TM
Carassius auratus LIFR
827 aa
TM
Gallus gallus LIFR
1083 aa
TM
Homo sapiens LIFR
1096 aa

Figure 6.7 Relative position and size of the four fibronectin type 3 domains identified in the goldfish, chicken and human LIFRs. TM represents the transmembrane region of the receptor. The number of amino acids composing each receptor is listed on the left. Identification of the conserved motifs was done using TMpred.

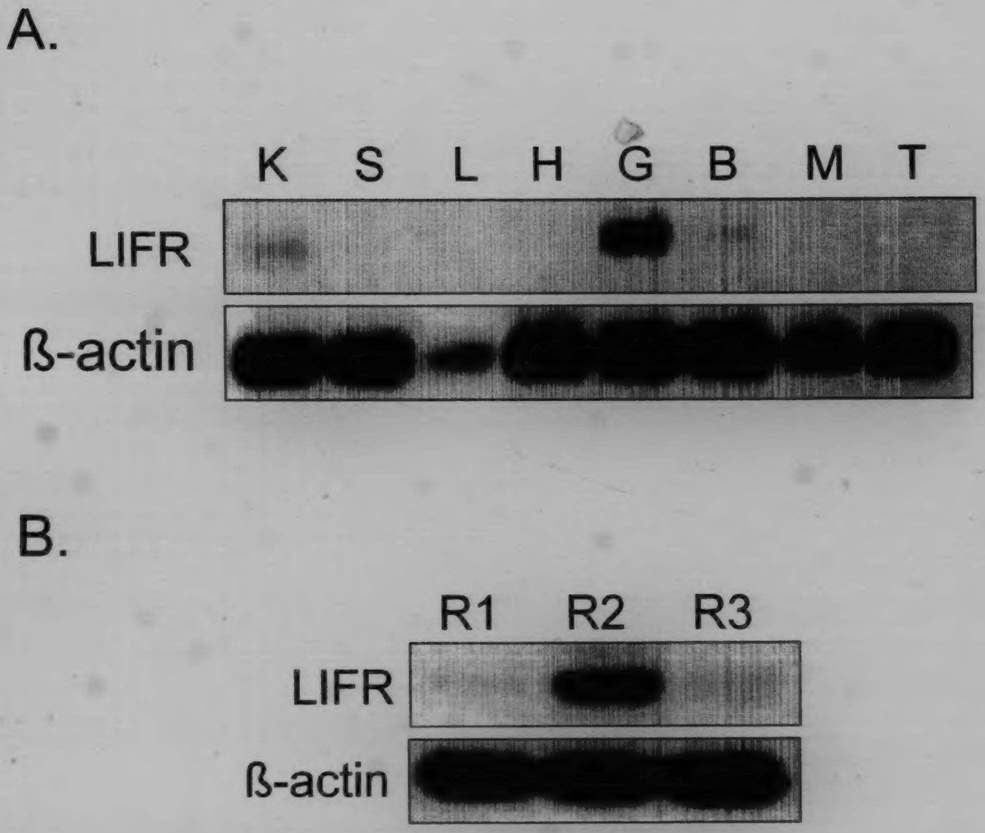

Figure 6.8 The mRNA levels of goldfish LIFR in tissues and sorted macrophages. The tissue expression of goldfish LIFR. (A) K-kidney, S-spleen, L-liver, H-heart, G-gill, B-brain, M-muscle, T-testes; (B) Expression of goldfish LIFR in progenitor cells (R1), macrophages (R2) and monocytes (R3), compared to a β-actin control. All RT-PCRs were run for 27 cycles. This experiment was repeated twice and the representative results are shown.

Chapter 7

Leukemia Inhibitory Factor and Leukemia Inhibitory Factor Receptor Regulate Neuronal and Embryonic Development of Zebrafish[1]

7.1 Introduction

Perhaps the most well studied aspect of LIF biology is its involvement in the survival, formation and repair of neurons and the maintenance of neural and embryonic stem cells [299-301]. LIF is commonly used to promote the self-renewal of mouse embryonic stem cells [302], mouse embryonic neural stem cells [299, 302-305], and human embryonic neural stem cells [306]. The mechanisms behind the retention of stem cell multipotency are not known, however LIFR/gp130 signalling has been shown to regulate the expression of Notch1 [307], which was shown to be important for the maintenance of neural stem cells [308]. Moreover, LIF expression is commonly associated with neural injury, and has been shown to be an essential stimulus for the proliferation of neuronal progenitor cells after injury [309]. In addition, LIFR/gp130 signalling was shown to be instrumental in the differentiation of neural progenitors into astrocytes [310].

Although LIF plays a central role in embryological development and pregnancy, LIF-/- mice have very few abnormalities aside from the observation that LIF-/- female mice failed to become pregnant [316, 331], a deficiency that was overcome by injection of LIF [332]. This observation suggests the central requirement for LIF *in vivo*.

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[1] A version of this chapter has been published:
Hanington et al, 2008. Analysis of leukemia inhibitory factor and leukemia inhibitory factor receptor in embryonic and adult zebrafish (*Danio rerio*). Developmental Biology. 314: 250-60.

Recently, an IL-6 family cytokine was identified in the carp [403]. This molecule, named M17, exhibited similar expression patterns to mammalian LIF and CNTF and shared closest amino acid identity to CNTF. In the previous chapter, I reported on the characterization of a goldfish M17 transcript and showed that it was highly expressed in the brain and kidney, and was able to induce the differentiation of goldfish monocytes into macrophages. The genomic analysis (see chapter 6) suggested that goldfish M17 was a teleost leukemia inhibitory factor (LIF). Furthermore, the analysis of zebrafish M17 suggested that it is syntenic to mouse and human LIF and OSM [48]. In addition, goldfish LIFR has also been identified, suggesting that the LIF/LIFR/gp130 signalling mechanism may be conserved in teleost fish [47].

The studies presented in this chapter outline the expression and function of zebrafish *m17* (called zebrafish *lif* here after) and zebrafish leukemia inhibitory factor receptor (*lifr*) during zebrafish embryogenesis and in the adult fish.

7.2 Experimental design

7.2.1 Analysis of zebrafish lif and lifr transcript expression

To determine the neurological and developmental roles *lif* and *lifr* play in the zebrafish, the expression pattern of zebrafish *lif* and *lifr* was assessed in both the tissues of the adult zebrafish and in different developmental stages of the zebrafish embryo. The relative expression of both *lif* and *lifr* was analyzed in the kidney, brain, liver, heart, spleen, and gill of adult fish, and in embryos 2, 12, 24, 48 and 72 hours post fertilization (hpf) using RT-PCR.

7.2.2 In situ analysis of lif and lifr expression

Understanding the exact location of *lif* and *lifr* expression during embryonic development was critical to being able to compare teleost and mammalian LIF functions. *In situ* hybridization was performed on 24, 48 and 72 hpf embryos using *lif* and *lifr*-specific mRNA probes that allowed for the visualization of the exact areas of expression and monitoring how the expression patterns of both transcripts changed during development.

7.2.3 Knockdown of lif and lifr message using morpholinos, and rescue of lifr morphants

Knockdown of the *lif* and *lifr* mRNA using morpholinos (short anti-sense RNA fragments that prevent translation of a mRNA transcript [541]) was important for understanding the functions of both transcripts. It was hypothesized that due to the high degree of redundancy observed in mammalian IL-6 family cytokines that knockdown of the *lif* transcript would have little impact upon the embryonic development. However, knockdown of the *lifr*, which is an important receptor for several members of the IL-6 cytokine family, would have a large impact on the development of the embryo and neurons. Morpholino experiments were repeated 6 times with hundreds of embryos each time in order to confirm the morphology and phenotype of the experimental and control embryos. To confirm that the knockdown phenotype observed was specific to the injected morpholino, mRNA rescues were done re-introducing *lifr* mRNA back into the *lifr*-injected embryo to rescue the morphant phenotype.

7.2.4 *Analysis of acetylated tubulin expression in lifr-MO embryos*

Acetylated tubulin is expressed in developing axonal projections and antibodies to it can be used to outline the synthesis of new neural projections. Observation of acetylated tubulin in the morpholino injected embryos allowed me to monitor the progression of the development of the nervous system and to visualize any defects in development caused by knockdown of the *lif* or *lifr* mRNA.

7.3 Results

7.3.1 *Expression of lif and lifr transcripts in zebrafish tissues and developing embryos*

Zebrafish *lif* and *lifr* transcript expression was assessed in the tissues of adult and developing embryos of zebrafish using RT-PCR. *lif* transcript expression was localized in the kidney and brain of adult zebrafish and was detectable as early as 12 hpf. *lif* transcript expression peaked between 24 and 48 hpf and remained high at 72 hpf (Fig 7.1). Adult zebrafish *lifr* transcript expression was observed in the kidney, gill, brain and spleen with lower expression levels observed in the liver. Like *lif*, *lifr* was expressed at 12 hpf and the expression gradually increased until it reached the highest levels observed, at 72 hpf (Fig. 7.1).

7.3.2 Genomic and chromosomal analysis of zebrafish lif and mammalian IL-6 family of cytokines

Leukemia inhibitory factor was found on zebrafish chromosome 5. This chromosome was searched for shared genes on chromosomes 11, 19, 7, and 3 of mice and chromosomes 22, 11, 9, 19, 7 and 16 of humans respectively, which contain members of the IL-6 cytokine family. Two *lif* genes have been identified on zebrafish chromosome 5, and they were closely flanked by myosin heavy polypeptide 1 on the posterior portion of the chromosome and by myosin heavy polypeptide 2 and double c2 beta on the anterior portion of the chromosome. Between the two M17 genes were calcium-binding protein 7 and zinc finger, matrin type 5 (Fig. 7.2A). Mouse chromosome 11 contained LIF and OSM in close proximity to each other, and anterior to these two genes were zinc finger, matrin type 5 and calcium binding protein 7. Further down the chromosome in the anterior direction is myosin heavy polypeptide 1 and 2 as well as double c2 beta (Fig. 7.2B). The human chromosome 22 had LIF and OSM in close proximity to each other. Posterior to these two genes were zinc finger, matrin type 5 and calcium binding protein 7. The myosin heavy polypeptides 1 and 2 and the double c2 beta genes were not found on this chromosome (Fig. 7.2C).

7.3.3 Phylogenetic analysis of zebrafish Lif and Lifr

Zebrafish Lif is closely related to goldfish, carp and puffer fish M17, an IL-6 cytokine I believe is similar to LIF based on functional and genomic analysis done in the goldfish and zebrafish [48]. The phylogenetic grouping containing M17 and zebrafish Lif appears to be related to mammalian CNTF, and the grouping of M17/zebrafish Lif and CNTF appear to be a basal group to all of the IL-6 family cytokines that bind to LIFR (Fig. 7.3). Zebrafish Lifr appears to be closely related to LIFRs of *Carassius auratus* (goldfish), *Ictalurus punctatus* (channel catfish), and *Tetraodon nigroviridis* (puffer fish). These fish LIFRs branch off from a common ancestor of the mammalian and chicken LIFRs, which are the fish LIFR's closest relative. The gp130 receptor subunit appears to be the ancestral receptor of the LIFRs and the oncostatin M receptors (OSMR). To date, no fish gp130 molecules have been identified, however, a zebrafish and a puffer fish OSMR have been identified indicating that the divergence of LIFR and OSMR may be traceable back to before the emergence of teleost fish (Fig. 7.3). Based on amino acid identity, zebrafish Lifr is most similar to goldfish LIFR (60%). Catfish LIFR (45% identity) and puffer fish LIFR (27% identity) appear to be its next closest relatives. Amino acid identity between zebrafish Lifr and mammalian and chicken LIFRs ranges from 19% to 21%. OSMRs and gp130 molecules share very little amino acid identity with zebrafish Lifr with between 19% to 24% and 15% to 17% identity, respectively (Fig. 7.4).

7.3.4 Developmental expression of the lif transcript

The developmental expression of *lif* at 24, 48 and 72 hpf was examined using *in situ* hybridization. At 24 hpf, a low-levels of *lif* expression in the fore-, mid- and hindbrain as well as the retina was observed (Fig. 7.5A, B). However, it remained unclear from the *in situ* hybridization whether or not *lif* was expressed throughout the CNS. This was likely because, at this developmental stage, *lif* transcripts were expressed at relatively low levels. At 48 hpf, the expression of *lif* was localized to the otic vesicle, retina, presumptive cranial sensory ganglia and the midbrain-hindbrain boundary (Fig. 7.5 C, F). The expression of *lif* at 72 hpf was similar to that observed at 48 hpf. Expression of *lif* was also observed in the otic vesicle, retina and cranial sensory ganglia. The expression in the retina appeared to be stronger at 72 hpf compared to earlier ages; *lif* was localized in 4 distinct clusters of ganglia, presumably the trigeminal (gV), facial (gVII), anterioventral ganglion (gAV) and anteriodorsal ganglion (gAD) (Fig. 7.5D). Interestingly, *lif* expression was not observed along the trunk muscles during the older developmental stages (Fig. 7.5E). The sense probe did not show any *in situ* staining indicating that there was no non-specific hybridization (Fig. 7.5G).

7.3.5 Developmental expression of the lifr transcript

The expression patterns of the *lifr* at 24, 48 and 72 hpf are shown in Fig. 7.6. *lifr* was strongly expressed in the notochord (NC), forebrain (F) and otic vesicle (OV) at 24 hpf (Fig. 7.6A-C). At 48 and 72 hpf, the expression of *lifr* was restricted to regions of the cranial ganglia, fore- and midbrain, as well as the retina (Fig. 7.6D, E). The sense probe did not show any *in situ* staining suggesting that there was no non-specific hybridization (Fig. 7.6F).

7.3.6 Functional analysis of Lif and Lifr in neuronal development

Previous studies have demonstrated a role for LIF and LIFR in neuronal development and survival [298, 513, 540]. To determine if these molecules possess a similar function in zebrafish embryonic development, we depleted embryos of Lif and Lifr protein using two antisense morpholino oligonucleotides (MOs) designed against the translation initiation sites of *lif* and *lifr* mRNA respectively. *lif* MO-injected embryos exhibit normal morphology at 48 hpf (Fig. 7.7B) when compared to wild type uninjected controls (Fig. 7.7A). In contrast, *lifr* MO-injected embryos display severe hydrocephaly at this stage (Fig. 7.7C).

lif and *lifr* were both expressed in the developing zebrafish hindbrain. To determine if these molecules were required for proper neural development in this area, we examined the cranial motor neurons of Lif-depleted and Lifr-depleted embryos using Isl1-GFP transgenic zebrafish embryos. These embryos express *gfp* under the control of a motor neuron-specific *isl1* gene enhancer [542]. *lif* MO-injected embryos do not exhibit any obvious defects in cranial motor neuron number or position (Fig. 7.7E), when compared to wild type controls (Fig. 7.7D). *lifr* MO-injected embryos, however, demonstrated a severe reduction in trigeminal (nV) and facial (nVII) branchiomotor neuron populations (Fig. 7.7F). Furthermore, the vagal (nX) motor neurons in *lifr* MO-injected embryos exhibited aberrant medio-lateral positioning and cell-to-cell spacing (Fig. 7.7F). The Isl1-GFP-expressing Rohon-beard cells and spinal motoneurons of *lifr* MO-injected embryos were indistinguishable from those of wild type embryos, indicating that unlike mammalian LIFR, teleost LIFR may not be associated with motor neuronal differentiation and development.

Given that *lif* and *lifr* are both expressed in cranial sensory ganglia, we analyzed the cranial axons of Lif-depleted and Lifr-depleted embryos using an antibody against acetylated tubulin, a protein that stains mature neurites [543]. The cranial axons of *lif* MO-injected embryos resembled those of wild type non-injected controls (Fig. 7.7G, H). *lifr* MO-injected embryos possess a decreased number of cranial axons (Fig. 7.7I). This was especially apparent in the tectum, where acetylated tubulin staining is nearly abolished in *lifr* morphants (Fig. 7.7I). *lifr* MO-injected embryos also exhibit extremely low levels of axon staining in the dorsal longitudinal fascicle and hindbrain neurons, but continued to demonstrate strong staining in axons of the anterior commissure neurons and trigeminal ganglion cells (Fig. 7.7 I).

To control for the specificity of the *lifr* antisense morpholino oligonucleotide, we attempted to rescue the *lifr* morphant phenotype by co-injecting *lifr* MO and *lifr* mRNA. The *lifr* mRNA has an altered sequence at the translation initiation site, which prevented it from binding to *lifr* MO. This synthetic mRNA continues to encode the normal Lifr amino acid sequence. Embryos injected with *lifr* mRNA alone did not exhibit any morphological phenotypes and did not appear to possess any defects in developing cranial ganglia, when compared to non-injected controls (Fig. 7.8 A, B). Ninety one per cent (*n*=86) of *lifr* MO-injected embryos exhibited hydrocephaly at 48 hpf (Fig. 7.8 C). Injection of *lifr* mRNA almost completely rescued this phenotype (Fig. 7.8 E), as only 8% (*n*=101) of *lifr* mRNA and *lifr* MO co-injected embryos exhibited hydrocephaly. Similarly, whereas 81% (*n*=64) of *lifr* MO-injected embryos had defects in branchiomotor neuron development (Fig. 7.8 D), only 97% (*n*=66) of *lifr* mRNA and *lifr* MO co-injected embryos (Fig 7.8 F) had normal morphology.

7.4 Discussion

Several studies have shown that the expression of LIF and of LIFR occur in distinct areas of the mammalian brain [544-548], and that these proteins are crucial for the survival, repair and formation of neurons as well as the maintenance of neural and embryonic stem cells [298-302, 513, 540, 549, 550]. Recently, LIF- and LIFR-like molecules have been identified in teleosts [48, 403, 551-553] and therefore, it is of much interest to assess the role of these proteins in fish. In this chapter, I report on the expression and the roles of *lif* and *lifr* in zebrafish. I found that (1) in adult zebrafish *lif* expression was restricted to kidney and brain, unlike that of *lifr* which was observed in the kidney, gill, brain, spleen and liver; (2) both *lif* and *lifr* were expressed as early as 24 hpf; and during embryogenesis *lif* and *lifr* were expressed in the retina, cranial sensory ganglia and the otic vesicles; and (3) while Lifr was essential for neuronal development and fish survival, its ligand Lif was not.

Zebrafish *lif* expression in the brain and the kidney was similar to that reported for other teleosts [48, 403]. Human LIF (hLIF) mRNA was shown to be transcribed in the liver, heart, brain, kidney, lung and thymus [554, 555]. Chicken LIF (chLIF) was shown to be expressed in the heart, liver, brain, kidney, thymus and spleen [496]. The expression profile of LIF in teleosts differed from that of the chLIF and hLIF and appeared to be more like the expression profile of CNTF [501], an observation that was supported by phylogenetic analysis that suggested that the zebrafish *lif* was closely related to mammalian CNTFs. Interestingly, the goldfish LIF homolog appears to function in a similar manner to mammalian LIF, and analysis of the genomic arrangement of zebrafish *lif* and chromosomal organization of goldfish LIF indicate that it is most similar to LIF or OSM [48, 551]. In addition, zebrafish Lif, M17 and MSH are the only putative LIFR-specific IL-6 family cytokines that have been identified in an ectothermic organism [48, 403, 551-553]. This suggests that zebrafish Lif may represent the functional ortholog of the LIFR-specific cytokines seen in mammals, and that OSM, CNTF and CT-1 may have developed from this ancestral molecule.

lif and *lifr* were not expressed significantly between 0 and 24 hpf. Consequently, it appears that Lif-Lifr signalling was not important for the early stages of zebrafish development. During the cleavage period (0-10 hpf) and prior to the segmentation period (12-22 hpf), there is rapid cell division, and I speculate that during this time frame, the maintenance of stem cells via Lif signalling is probably not required. However, during the segmentation and hatching period (48-72 hpf), organogenesis is complete and there may be a requirement for Lif/Lifr for the maintenance of stem cells of the various tissues. To my knowledge, this is the first report on the expression of *lif* in the otic vesicle, where it may play a role in the development of the vestibulo-acoustic system.

LIF has been shown to possess multiple influences on the nervous system including the survival of neurons. Recently, LIF has been extensively studied for its potential role as an environmental signal regulating retinal development. LIF strongly affects neurogenesis of the vertebrate retina. In mouse retina, LIF inhibited rod photoreceptor cell differentiation and was shown to promote bipolar cells differentiation [556, 557]. These functions suggest that LIF helps in fine-tuning the balance between photoreceptors and other retinal cell types and hence plays a key role in the development of the retinal architecture for proper vision. In developing nervous system, LIF promotes the survival of embryonic sensory neurons [558, 559]. For example, LIF was shown to act on developing cranial sensory neurons such as the trigeminal ganglia by promoting their survival [560]. It is likely that Lif may have similar effects in these regions in the developing zebrafish. However, I found that Lif-depleted zebrafish developed normally and had no indications of any neural defects. My results are consistent with several studies in which LIF knockouts had no signs of phenotypic abnormality or neural defects [331, 558, 561]. Thus, my findings suggest that the role of Lif may be redundant in terms of zebrafish neural development and that it can be compensated for by the remaining LIFR/gp130 binding cytokines, possibly CNTF, CT-1 or OSM if they exist in fish, or the M17 homolog (MSH). Consistent with this suggestion, it was shown that neuronal deficits in mice are only observed when both the LIF and CNTF genes are knocked out [562].

LIFR has been shown to be involved in neuronal formation and survival [513, 540]. Here I show that Lifr knockdown generated distinct phenotypes relevant to cranial ganglia development, tectum development, branchiomotor development, and vagal motor neuron positioning. These findings not only suggest that Lifr signalling was important for neuronal development in zebrafish but show that the receptor was the essential component for this process. *lifr* morphants also die within 6 days post-fertilization which may be due to the disruption of vital multiple organ systems affected by the knockdown [563]. I also observed partial reduction in the cranial axons, suggesting that some cranial neurons use different signalling pathways in their proliferation, differentiation, maintenance and survival. Motor neuron development was shown to be severely affected in LIFR-deficient mice [558, 563, 564]. However, the *lifr* morphants did not show detectable loss of spinal motor neurons. These findings suggest that Lifr signalling was not involved in motor neuronal differentiation and maintenance during zebrafish development. It will be interesting to determine whether zebrafish motor neurons are affected in gp130-deficient zebrafish.

The *lif* expression pattern did not fully match that of *lifr* during embryogenesis and in adult fish. During mouse embryogenesis, LIFR exhibited slight differences in spatiotemporal expression patterns compared to LIF [565]. To date no distinct cell types, organs or tissues have been recognized as the main producers of the LIF protein. However, the signalling through the LIFR complex was shown to be primordial for maintenance of cells pluripotency [565].

In conclusion, this chapter characterized the expression of Lif/Lifr during zebrafish embryogenesis and in the tissues of adult zebrafish. I provided evidence that adult zebrafish express *lif* in the brain and kidney, and that embryonic fish express *lif* in the otic vesicle, retina and cranial sensory ganglia. I also showed that *lifr* was expressed in the zebrafish kidney, gill brain and spleen and was detectable as early as 12 hpf in the developing embryo. Lifr was also found to be crucial for proper neural development in zebrafish.

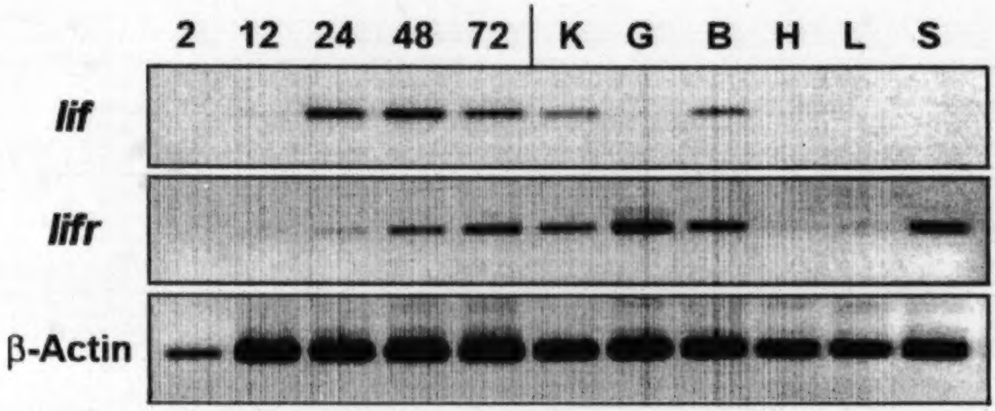

Figure 7.1 The mRNA expression of zebrafish *lif* and *lifr* in tissues and embryos. RT-PCR showing expression of *lif* and *lifr* in zebrafish embryos 2, 12, 24, 48 and 72 hours post fertilization, and in the kidney (K), gill (G), brain (B), heart (H), liver (L) and spleen (S) of adult zebrafish. *Lif* and *lifr* expression was compared to a β-actin loading control.

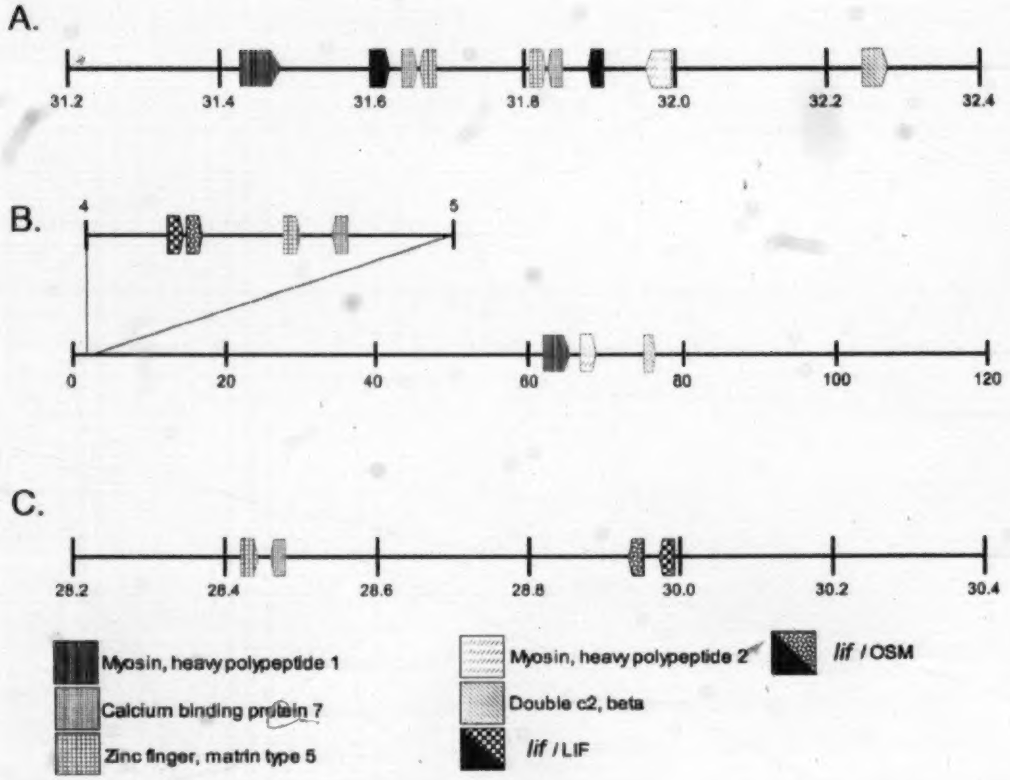
A.
31.2
31.4
31.6
31.8
32.0
32.2
32.4
B.
4
5
0
20
40
60
80
100
120
C.
28.2
28.4
28.6
28.8
30.0
30.2
30.4
Myosin, heavy polypeptide 1
Calcium binding protein 7
Zinc finger, matrin type 5
Myosin, heavy polypeptide 2
Double c2, beta
lif / LIF
lif / OSM

Figure 7.2 Synteny analysis of zebrafish *lif*. Synteny analysis of M17 genes located on zebrafish chromosome 5 (A), mouse chromosome 11 (B) and human chromosome 22 (C). Numbers represent the location within the chromosome in millions of base pairs. Two zebrafish M17 genes have been identified and are differentially labelled to distinguish between the two.

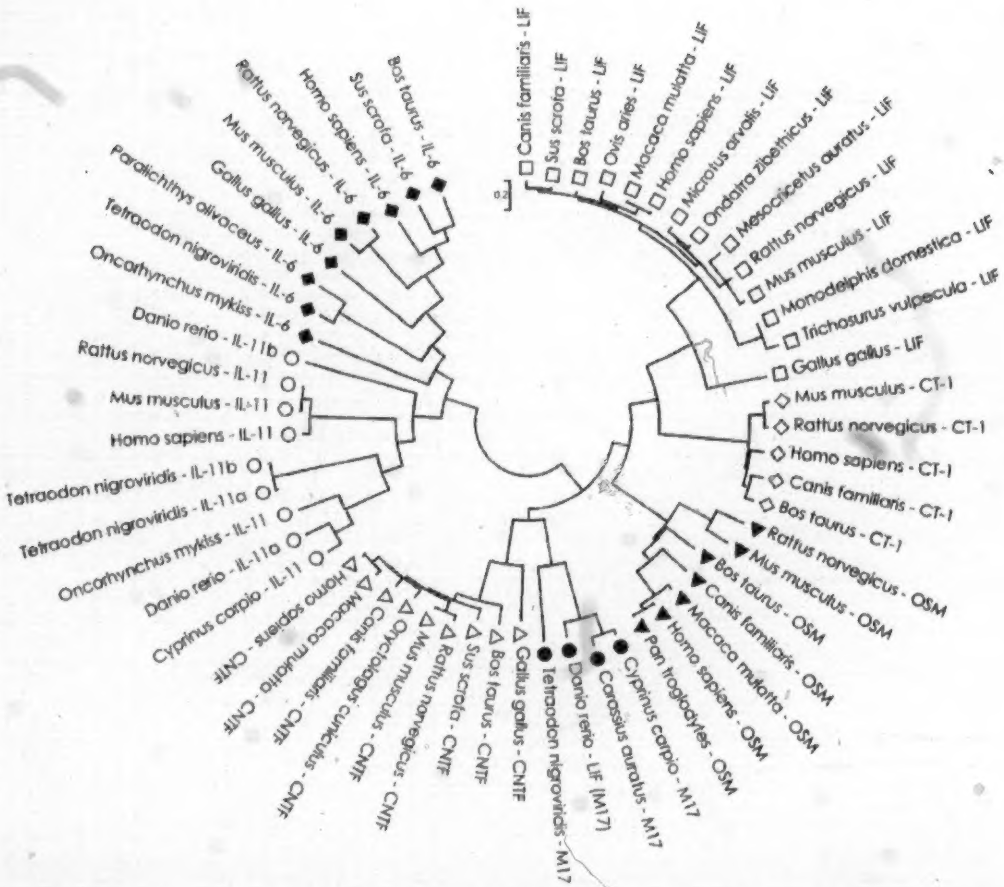
0.2
Canis familiaris - LIF
Sus scrofa - LIF
Bos taurus - LIF
Ovis aries - LIF
Macaca mulatta - LIF
Homo sapiens - LIF
Microtus arvalis - LIF
Ondatra zibethicus - LIF
Mesocricetus auratus - LIF
Rattus norvegicus - LIF
Mus musculus - LIF
Monodelphis domestica - LIF
Trichosurus vulpecula - LIF
Gallus gallus - LIF
Mus musculus - CT-1
Rattus norvegicus - CT-1
Homo sapiens - CT-1
Canis familiaris - CT-1
Bos taurus - CT-1
Rattus norvegicus - OSM
Mus musculus - OSM
Bos taurus - OSM
Canis familiaris - OSM
Macaca mulatta - OSM
Homo sapiens - OSM
Pan troglodytes - OSM
Cyprinus carpio - M17
Carassius auratus - M17
Danio rerio - LIF (M17)
Tetraodon nigroviridis - M17
Gallus gallus - CNTF
Bos taurus - CNTF
Sus scrofa - CNTF
Rattus norvegicus - CNTF
Mus musculus - CNTF
Oryctolagus cuniculus - CNTF
Canis familiaris - CNTF
Macaca mulatta - CNTF
Homo sapiens - CNTF
Cyprinus carpio - IL-11
Danio rerio - IL-11a
Oncorhynchus mykiss - IL-11
Tetraodon nigroviridis - IL-11a
Tetraodon nigroviridis - IL-11b
Homo sapiens - IL-11
Mus musculus - IL-11
Rattus norvegicus - IL-11
Danio rerio - IL-11b
Oncorhynchus mykiss - IL-6
Tetraodon nigroviridis - IL-6
Paralichthys olivaceus - IL-6
Gallus gallus - IL-6
Mus musculus - IL-6
Rattus norvegicus - IL-6
Homo sapiens - IL-6
Sus scrofa - IL-6
Bos taurus - IL-6

Figure 7.3 Phylogenetic tree showing the relationships between LIF, M17, OSM, CNTF, CT-1, IL-6 and IL-11 from a number of different vertebrate species. Zebrafish *lif* groups together with M17 molecules of the goldfish, carp and pufferfish. These molecules appear to be phylogenetically related to the CNTFs of mammals and together with CNTF appear to represent the basal group of the LIFR-specific IL-6 family cytokines. The phylogenetic tree was generated using the neighbour-joining method and was bootstrapped 10000 times to increase confidence and no bootstrap value was lower than 57%. Open circle (○) - IL-11, Open square (□) - LIF, close circle (●)-M17, close triangle (▼)- OSM, close diamond (♦)- IL-6.

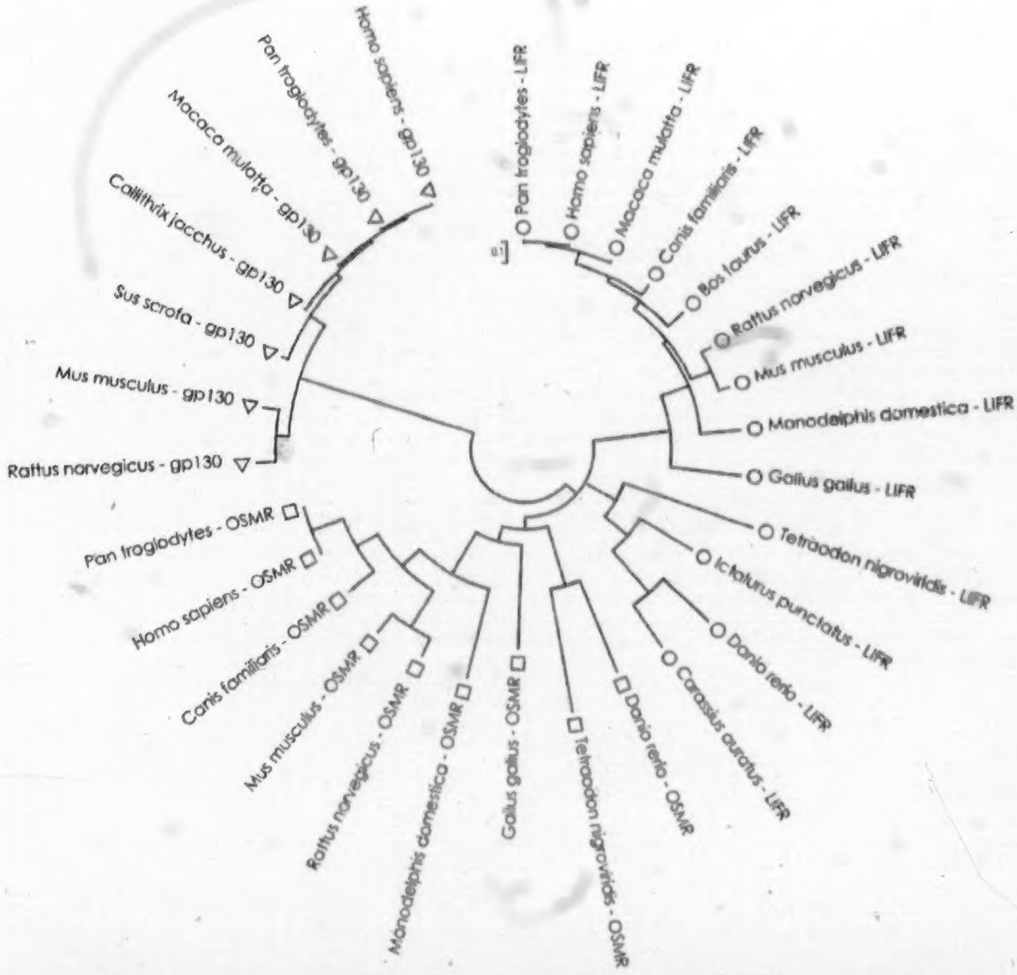
Homo sapiens - gp130 △
Pan troglodytes - gp130 △
Macaca mulatta - gp130 △
Callithrix jacchus - gp130 ▽
Sus scrofa - gp130 ▽
Mus musculus - gp130 ▽
Rattus norvegicus - gp130 ▽
Pan troglodytes - OSMR □
Homo sapiens - OSMR □
Canis familiaris - OSMR □
Mus musculus - OSMR □
Rattus norvegicus - OSMR □
Monodelphis domestica - OSMR □
Gallus gallus - OSMR □
□ Tetraodon nigroviridis - OSMR
□ Danio rerio - OSMR
○ Carassius auratus - LIFR
○ Danio rerio - LIFR
○ Ictalurus punctatus - LIFR
○ Tetraodon nigroviridis - LIFR
○ Gallus gallus - LIFR
○ Monodelphis domestica - LIFR
○ Mus musculus - LIFR
○ Rattus norvegicus - LIFR
○ Bos taurus - LIFR
○ Canis familiaris - LIFR
○ Macaca mulatta - LIFR
○ Homo sapiens - LIFR
○ Pan troglodytes - LIFR

Figure 7.4 Phylogenetic tree showing the relationships between LIFR, OSMR and gp130 molecules of different vertebrates. The *lifr* of zebrafish (shown at bottom) groups closest to the other known fish LIFR molecules, with the highest similarity to goldfish LIFR. The phylogenetic tree was generated using the neighbour-joining method and was bootstrapped 10,000 times to increase confidence and no bootstrap value was lower than 71%. Open circle (○) - LIFR, Open square (□) - OSMR, open triangle (△)- gp130.

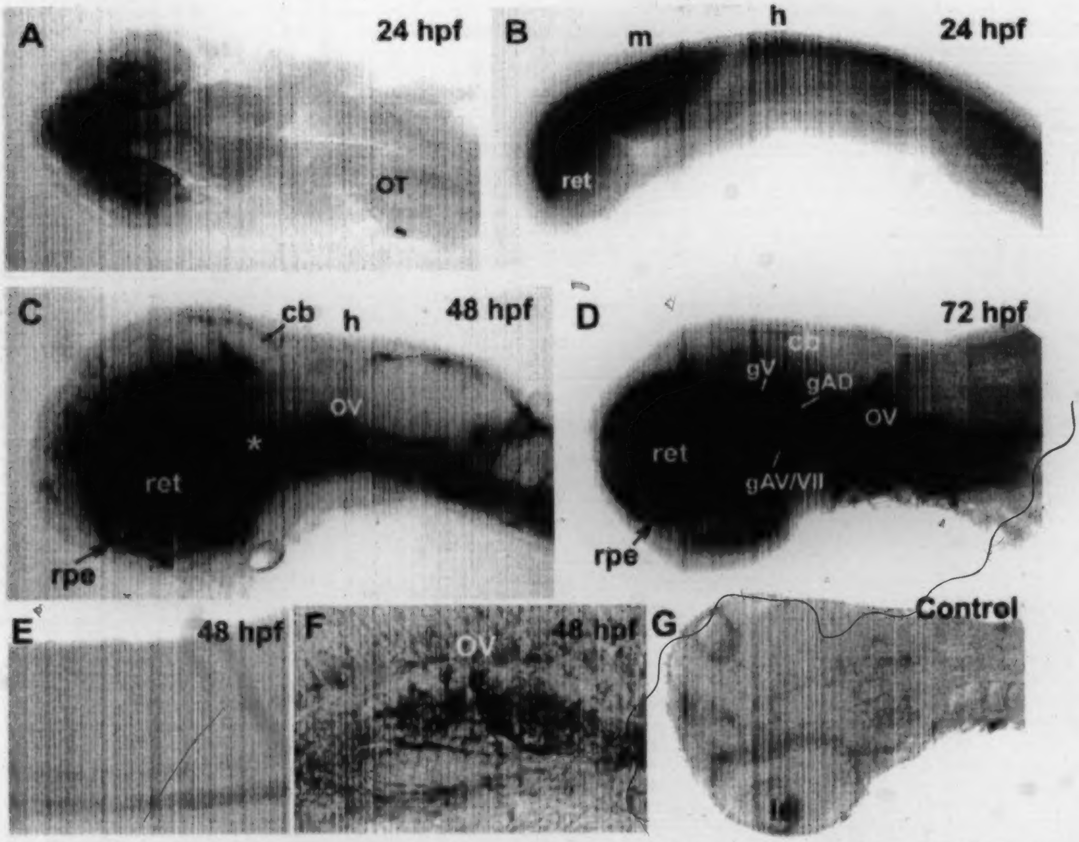

Figure 7.5 Expression patterns of *lif* in developing zebrafish embryos. Embryonic and larval expression patterns of *lif*. A, B: *lif* expression was observed to be most prominent in the retina, however expression was widespread and there was no exclusive expression of *lif* in the distinct areas of the brain. C: At 48 hpf, *lif* was clearly visible in regions of the cranial sensory ganglia (asterisk, *), otic vesicle (OV), retina (ret) and area of the midbrain-hindbrain boundary. D: At 72 hpf, *lif* was present at relatively high levels in the retina, otic vesicle and the cranial sensory ganglia. *lif* was localized in the presumptive trigeminal (gV), facial (gVII), anterioventral ganglion (gAV) and anteriodorsal ganglion (gAD). E: *lif* was not expressed in muscle and trunk neurons. F: Expression of *lif* in the otic vesicle was visible at both 48 hpf and 72 hpf. G: *In situ* hybridization pattern of the *lif* sense probe. (t), telencephalon; (m), midbrain; (h), hindbrain; (cb), cerebellum; (rpe), retinal pigmented epithelium; (le), lens.

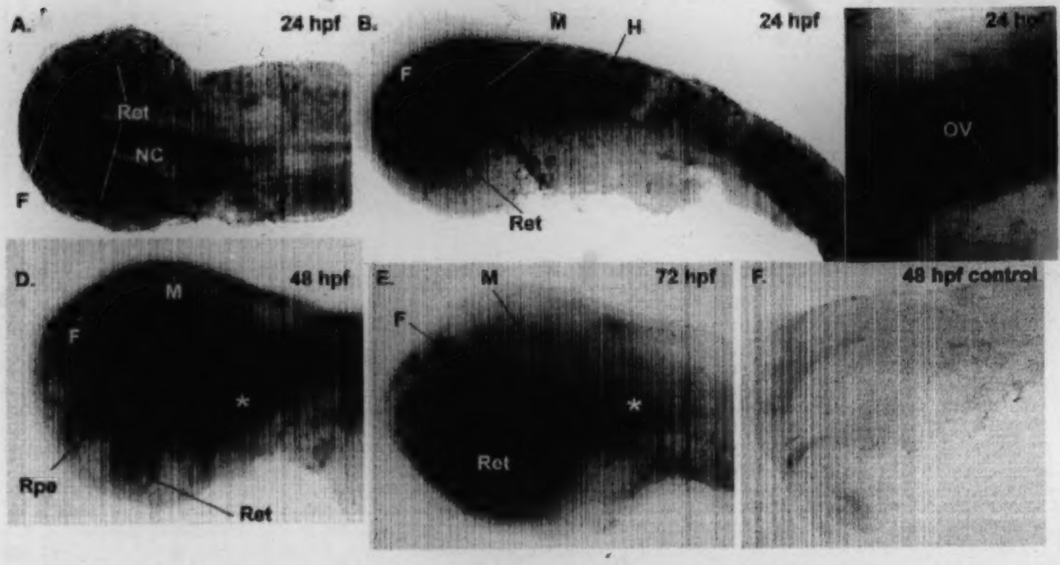
A.
24 hpf
Ret
NC
F
B.
M
H
24 hpf
F
Ret
24 hpf
OV
D.
48 hpf
M
F
*
Rpe
Ret
E.
M
72 hpf
F
*
Ret
F.
48 hpf control

Figure 7.6 Expression patterns of *lifr* in developing zebrafish embryos. Embryonic and larval expression patterns of *lifr*. A, B: At 24 hpf, *lifr* was highly expressed in the forebrain, midbrain and notochord. C: *lifr* was also visible in the otic vesicle (OV) at 24 hpf. D: At 48 hpf, *lifr* was localized in regions of the cranial sensory ganglia (asterisk, *), retina and forebrain. E: Expression pattern of *lifr* at 72 hpf was similar to that at 48 hpf. *lifr* was expressed in regions of the cranial sensory ganglia (*) and forebrain. F: *lifr* was not expressed in trunk muscle and trunk neurons. (f), forebrain; (m), midbrain; (h), hindbrain; (cb), cerebellum; (rpe), retinal pigmented epithelium.

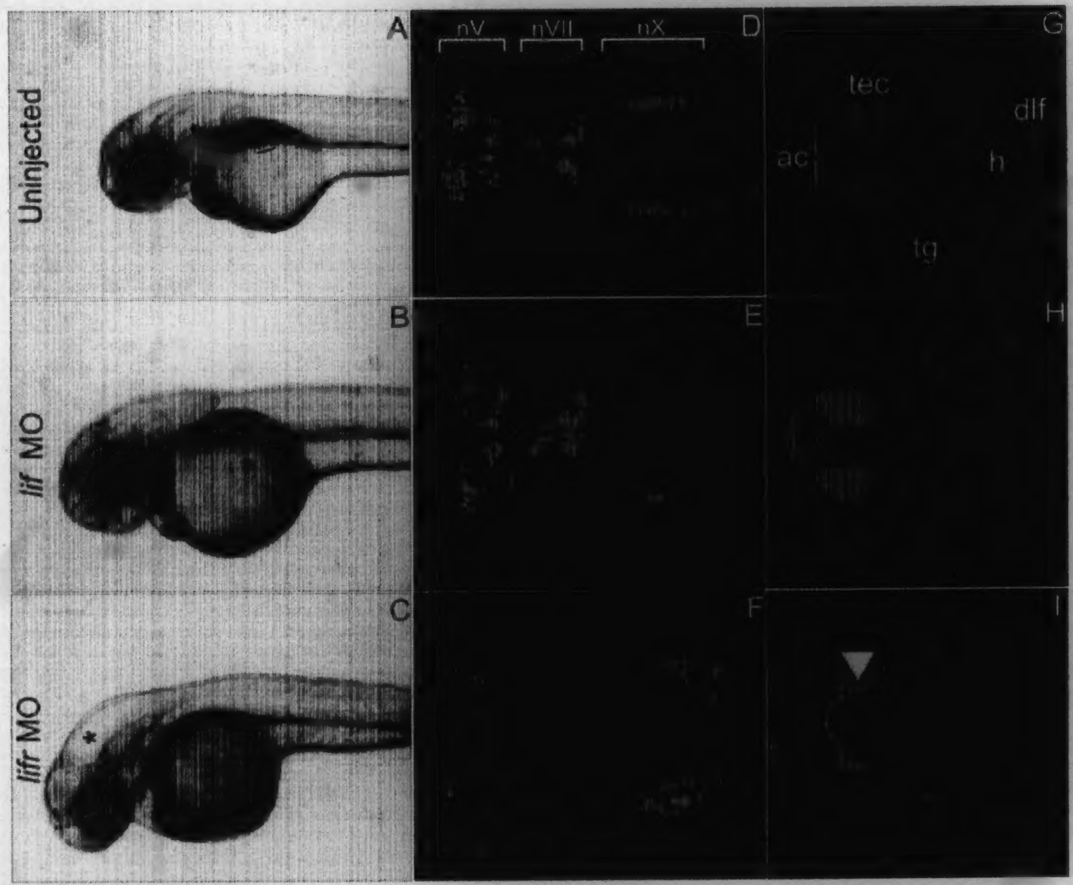

Figure 7.7 Morpholino knockdown of *lif* and *lifr* in developing zebrafish embryos. *lifr*-depleted, but not *lif*-depleted embryos exhibit hydrocephaly and neural defects. A, B, C: Live images of 48 hpf embryos in lateral view with anterior to left. Embryos are wild type uninjected (A), injected with *lif* MO (B), or injected with *lifr* MO (C). Asterisk (*) marks hydrocephaly observed in *lifr* morphants, but not in *lif* MO-injected or uninjected embryos. D, E, F: Confocal fluorescent composite 20X images of hindbrain branchiomotor neurons in wild type uninjected (D), *lif* MO-injected (E), or *lifr* MO-injected (F) 48 hpf Isl1-GFP transgenic embryos. View is dorsal with anterior to left. G, H, I: Confocal fluorescent composite 20X images showing anti-acetylated antibody-stained cranial axons of 48 hpf embryos. View is dorsal with anterior to left. Embryos are wild type uninjected (G), *lif* MO-injected (H), or *lifr* MO-injected (I). Arrowhead indicates significant loss of staining in the tectum of *lifr* morphants. (tec), tectum; (ac), anterior commissure neurons; (dlf), dorsal longitudinal fasciculus; (h), hindbrain; (tg), trigeminal ganglion.

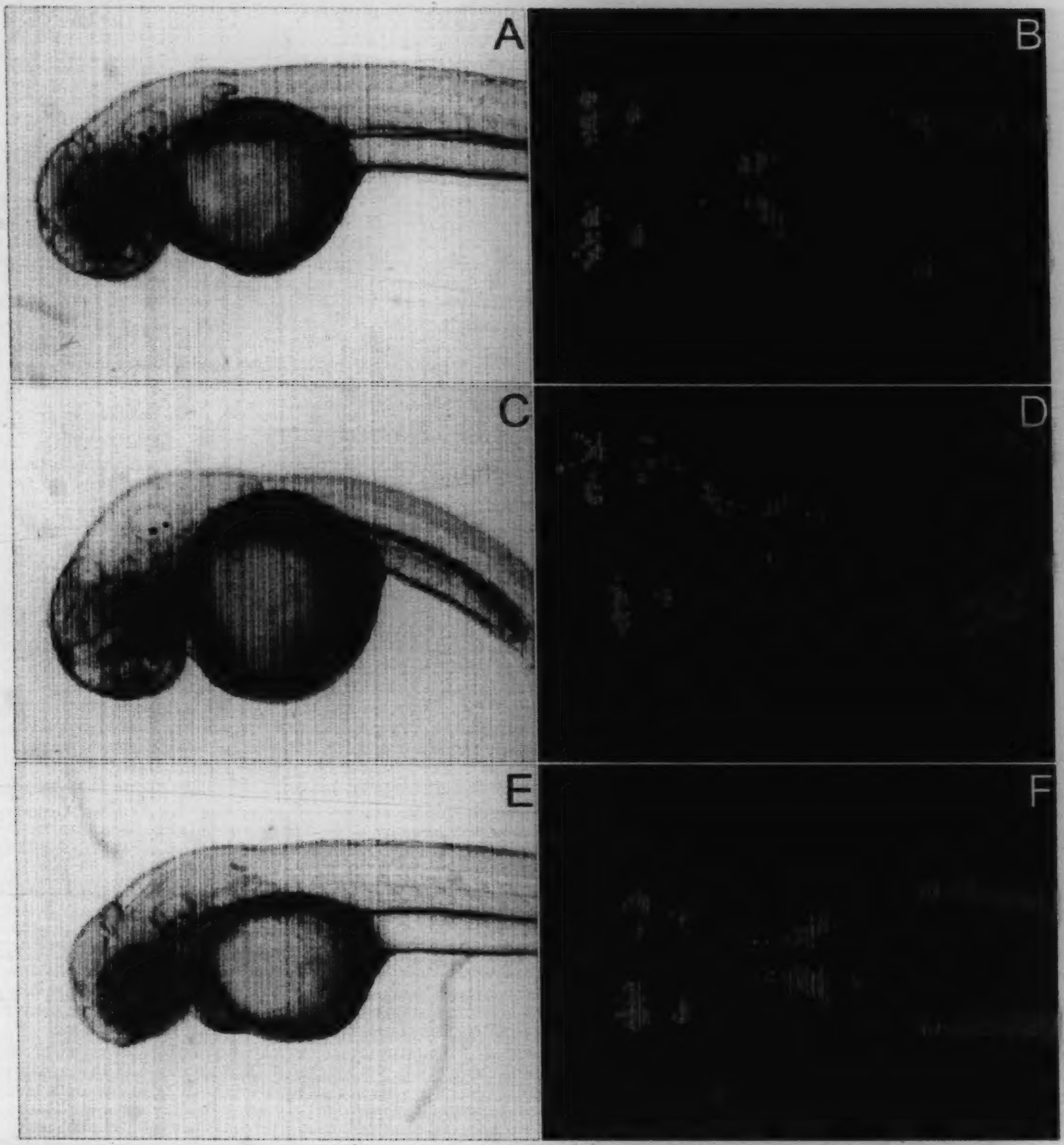

Figure 7.8 Rescue of the *lifr* MO phenotype by co-injection of the *lifr* MO with *lifr* synthetic mRNA. Non-injected embryos exhibited no morphological defects (A) and developed normal cranial ganglia (B) at 48 hpf. In contrast, *lifr* MO injected embryos exhibited severe hydrocephaly (C) and possess several defects in branchiomotor neuron development (D) at 48 hpf. Co-injection of *lifr* MO with *lifr* mRNA rescued the *lifr* MO phenotype and resulted in ~ 92% of embryos displaying a normal morphological phenotype (E), and ~97% of embryos developing normal branchiomotor neurons (F).

Chapter 8

General Discussion

Fish have remained the most successful vertebrates since their theorized beginnings 510 million years ago [566]. To date, over 32,000 species of fish currently exist, with the possibility of thousands of species yet to be discovered [567, 568]. Their success comes in part from being able to successfully protect themselves from infection and injury, a difficult task considering the easy transmission routes for infectious agents provided by water. Understanding the fish immune response and hematopoiesis is important not only from an evolutionary perspective, but also because during their long existence fish have been extremely successful and have developed several elegant strategies for dealing with immune challenges and maintenance of homeostasis. It is not surprising, therefore, that the field of comparative (fish) immunology and hematopoiesis research is expanding very rapidly. A number of fish model systems have been developed such as the goldfish/carp, zebrafish, catfish, pufferfish and rainbow trout, These models represent key groups among the diversity of teleost fishes. Due to the extensive time that fish have had to evolve, a great deal of diversity can be seen even within the bony fish lineage. In fact, the evolutionary distance between two teleost species the rainbow trout (*Oncorhynchus mykiss*) and the carp (*Cyprinus carpio*) is substantially larger than the distance between mice and humans [569].

Fish hematopoiesis research has been spearheaded by groups focusing on two cyprinid model organisms: the zebrafish which has yielded a vast amount of genetic data on the regulation of cell development, and the goldfish/carp which has been used to demonstrate functional control of cell development in fish. The research on zebrafish has produced several findings that suggest teleost and mammalian hematopoiesis are under very similar transcriptional control [33, 65]. Zebrafish have become essential research organisms for the discovery and characterization of genetic markers for cells of all types and their developmental stages. As a result, zebrafish have become a popular model for studying defects in cell cycle regulation and cancer. Furthermore, zebrafish, carp and goldfish studies in which donor kidney cells are transplanted into recipient fish, suggest that developmental processes that drive functional leukocyte development are also driven by similar pathways to those of the mammalian immune cell development [40-42]. Thus, the results obtained using these cyprinid species suggest that the general processes of hematopoiesis have not changed significantly over the evolutionary distance between fish and mammals. These data imply that the selective pressures on fish and mammals have not required the development of drastic alternative immune effectors or processes. However, these findings are contrasted by observations that indicate that although the end result may be the same, the mechanisms that control hematopoiesis in fish are different from those of mammals. As stated earlier in this thesis, fish cells are able to produce their own growth factors [24, 44, 434], and possess unique mechanisms for regulating their own development, such as the soluble CSF-1R receptor or and a unique short form of granulin that is expressed only in hematopoietic tissues [46, 49]. Consequently, fish cells are able to become immortal seemingly spontaneously without any transformation process, which is a requirement for mammalian cells [461, 570].

In this thesis, I described work done to extend our understanding of teleost hematopoiesis, using as a model organism, the goldfish. I chose to identify and functionally characterize growth factors whose presence in the goldfish was suggested in studies aimed at understanding goldfish myelopoiesis. These observations can be grouped into three as follows: (A) *In vitro* kidney derived primary kidney macrophage cultures exhibited spontaneous growth *in vitro* for a finite period before reaching a phase of growth characterized by cell clumping and apoptosis. The supernatants from established macrophage cultures enhanced the proliferation of newly established cultures, whereas the supernatants from dying cultures induced apoptosis when added on their own or abrogate proliferation of primary macrophages when added in combination with proliferation-inducing culture supernatants. Thus, the primary kidney macrophages produced their own growth factors and appeared to employ novel regulatory mechanisms that controlled their growth and development [24, 44, 49]. (B) Goldfish kidney homogenates enriched for mononuclear cells are predominantly composed of relatively small cell population immediately after isolation. These small cells, when analyzed by flow cytometry for their size and internal complexity characteristics, were smaller than monocytes, macrophages and granulocytes, and had relatively simple internal complexity. The analysis of cell subpopulations during cultivation of primary kidney leukocytes enriched for mononuclear cells indicated that that these small cells eventually developed into monocytes and macrophages, suggesting that they may represent the progenitor cell pool [24, 44, 53]. (C) In addition to the classical differentiation pathway whereby semi-committed progenitor cells developed into blood monocytes and then into tissue macrophages upon exposure to tissue microenvironments, it was observed that the sorted progenitor cell population was also capable of rapid differentiation and development into mature macrophages in culture [44, 53]. This 'alternative' differentiation pathway suggested that the progenitor cell population was composed, at least in part, of cells that were influenced by growth factors that could support different developmental pathway of monopoiesis. The 'alternative' differentiation observed was found to be akin to embryonic hematopoietic events observed in foetal mammals and in embryonic zebrafish where it was termed primitive hematopoiesis [33].

The identification and functional characterization of goldfish CSF-1 [49, 50, 243], granulin [45, 46, 293] and LIF [47, 48, 405] done during my thesis research has furthered our understanding of the goldfish PKM culture system [24, 44], which was reliant on non-defined cell conditioned medium to drive goldfish macrophage development *in vitro*.

Macrophage colony-stimulating factor was assumed to exist in fish based solely on the knowledge that its receptor was present [49, 241, 242]. Its identification in the goldfish, zebrafish and rainbow trout [50, 243] was essential for understanding myelopoiesis in fish. Although the functional assessment of goldfish CSF-1 indicated that it functioned very much like its mammalian homolog, molecular evidence suggested that fish possessed distinct translated splice variants from those of mammals which has implications on the possible functions of fish CSF-1 [243]. Importantly, using recombinant CSF-1 to stimulate proliferation and differentiation of fish monocytes and macrophages demonstrated that fish CSF-1 was involved in the regulation of classical monopoiesis [50].

Preliminary analysis of CSF-1 biology in the goldfish stemmed from studies characterizing the CSF-1 receptor, as well as a novel soluble form of the receptor that is not present in mammals [49]. The soluble CSF-1R had been shown to be able to abrogate the proliferation-inducing effects of CCM [49] indicating that it was a novel regulatory mechanism employed by fish to retard monocyte/macrophage development. The soluble receptor was highly expressed in the progenitor cell population suggesting that it was possibly a mechanism utilized by the progenitor cells to prevent unwanted differentiation into monocytes via exposure to monocyte/macrophage produced CSF-1 [49, 50]. The identification of goldfish CSF-1 allowed me to revisit the sCSF-1R regulatory mechanism specifically using rgCSF-1 to stimulate monocyte proliferation. Using rgCSF-1 in combination with the sCSF-1R I was able to abrogate rgCSF-1-induced monocyte proliferation, demonstrating that the sCSF-1R is a CSF-1-specific regulator of monocyte/macrophage development in fish [50]. Interestingly, the supernatants of goldfish CCL-71 fibroblasts also possess growth factors that I have shown to be primarily CSF-1, by the abrogation of proliferative effects using sCSF-1R. This mirrors mammalian macrophage biology in which fibroblast feeder cell (commonly L-929 fibroblasts) supernatants are used to supplement macrophage cultures [433]. Moreover, it has been shown that mammalian monocytes can produce biologically relevant levels of CSF-1 after activation [571, 572]. Thus, it is possible that mammalian monocytes were once able to produce CSF-1 without being activated, indicating that, at one point in time mammalian monocytes/macrophages may have been able to regulate their own development akin to what I have demonstrated to be the case for the goldfish.

Granulin was identified as a growth factor because it was associated with proliferating PKM cultures (i.e., significant up regulation of granulin mRNA in proliferating macrophages). Involvement of granulins in proliferation has been previously reported; mammalian progranulin has been shown to be a potent inducer of proliferation in a number of cell types [249]. Goldfish granulin proved to be unique in that it was associated with hematopoietic tissues only and was significantly smaller than the mammalian progranulin molecule or the recently identified zebrafish progranulin molecules [253]. Importantly, goldfish granulin regulated the proliferation of progenitor cells *in vitro* and influenced monocyte development. Interestingly, the highest granulin expression in the PKM cultures was in monocytes, which were inhibited from differentiating into macrophages by granulin. Furthermore, granulin was detected in the supernatants of proliferating goldfish PKM cultures indicating that it represented one of the endogenous growth factors produced by the PKM [46]. The characterization of goldfish granulin was important as it was the first growth factor that I identified that was produced by the goldfish PKM. Its functions support the hypothesis that fish macrophages may have unique developmental mechanisms [46, 49].

LIF was originally identified as M17 in the carp [403] and was the first IL-6 family cytokine to be functionally characterized in fish [48]. Based on molecular analysis, M17 was hypothesized to be a fish homolog of mammalian LIF, a growth factor that had been shown to have a wide range of functions, the most studied being its ability to maintain the pluripotency of stem cells [298, 313]. I have shown that goldfish LIF functions much like mammalian LIF, because it induced macrophage differentiation and activation, and induced the proliferation of progenitor cells [48]. These data combined with analyses of the LIFR in the goldfish [47] and the zebrafish [405] demonstrated that LIF/LIFR was critical for important developmental processes, including development of immune cells and those of the nervous system, both of which have been shown to be LIF/LIFR functions in mammals [298, 299, 304, 573]. Interestingly, goldfish LIF was not able to retain the pluripotency of the goldfish progenitor cell population *in vitro,* indicating that either this functional property was not shared between fish and mammalian LIF, or that the *in vitro* cultured goldfish progenitor cell population was already committed to a specific lineage pathway to be considered pluripotent.

Research on developmental pathways and controlling mechanisms is dependent on having markers for different cell stages and on having homogenous cell populations to work with in order to be able to examine specific developmental effects. I believe that the functional characterization of different growth factors done in my thesis research significantly reduced the heterogeneity of the goldfish macrophage *in vitro* model system, allowing for the systematic gathering of transcript expression information and building of a concrete database of possible cell stage markers. For example, recombinant CSF-1-treated PKM cultures appeared to be more homogeneous long-term cultures when compared to CCM-grown cultures, which will allow for more accurate functional assessment of goldfish macrophages. Furthermore, the identification of goldfish macrophage growth factors and their receptors has increased the pool of potential markers for the monocyte and macrophage of fish. These markers will be useful because they are not limited to only being genetic markers, and also because the antibodies that recognize these molecules will be of central importance for the analysis of macrophage developmental and functional mechanisms in teleosts.

A powerful technique used in mammalian immunology is the transplantation of healthy stem cells into lethally irradiated recipient organisms for monitoring of stem cell development *in vivo*. This technique has now been used in both the zebrafish [574] and carp/goldfish [40-42] model systems. Using both the zebrafish (in which transgenic GFP labelled cells were transplanted into recipient fish) or the carp/goldfish (in which gynogenetically bred tetraploid offspring kidney cells were transplanted into anaemic or irradiated triploid parental recipients), it was demonstrated that the anterior and posterior portions of the fish kidney contained pluripotent stem cells that were capable of re-populating all the blood cell types of the fish [40, 41, 574, 575]. Unfortunately, neither of these two independent studies used any functional cell markers to identify the re-populating cell populations.

Work done in this thesis and by others [32, 33, 36], has begun to not only identify the macrophage developmental markers, but also provide *in vitro* data on growth factor functions in fish. Studies presented in this thesis regarding goldfish CSF-1, granulin and LIF provide the baseline framework for the examination of fish macrophage development *in vivo.* Since one out of every million to ten million cells were reported to be pluripotent stem cell in mammals [576], and if this ratio holds true in fish, then *in vitro* studies of stem cell biology will be challenging due to an infinitely small number of stem cells that can be isolated from a kidney of individual fish. The availability of specific cell markers and recombinant growth factors and their receptors may allow for the development of cell enrichment protocols enabling researchers to study fish stem cell biology *in vitro.* Alternatively, the analysis of stem cell developmental potential and their characteristics at distinct stages of lineage commitment, may be done by the types of *in vivo* experiments described above using zebrafish and carp/goldfish model systems, and specific macrophage markers that I have begun to characterize in my work.

The characterization of each of the goldfish macrophage growth factors represents the first comprehensive functional analysis of these factors in fish. I believe that my studies have provided the foundation for future analyses of myelopoiesis not only in the goldfish but also for other teleosts. Individually, each of these growth factors has unique properties that influence myeloid proliferation, differentiation and activation (Fig. 8.1). Of course, in a biological system these growth factors would work in concert with each other and with other cytokines to orchestrate the overall hematopoietic needs of an organism for the maintenance of homeostasis or responses to sudden insults.

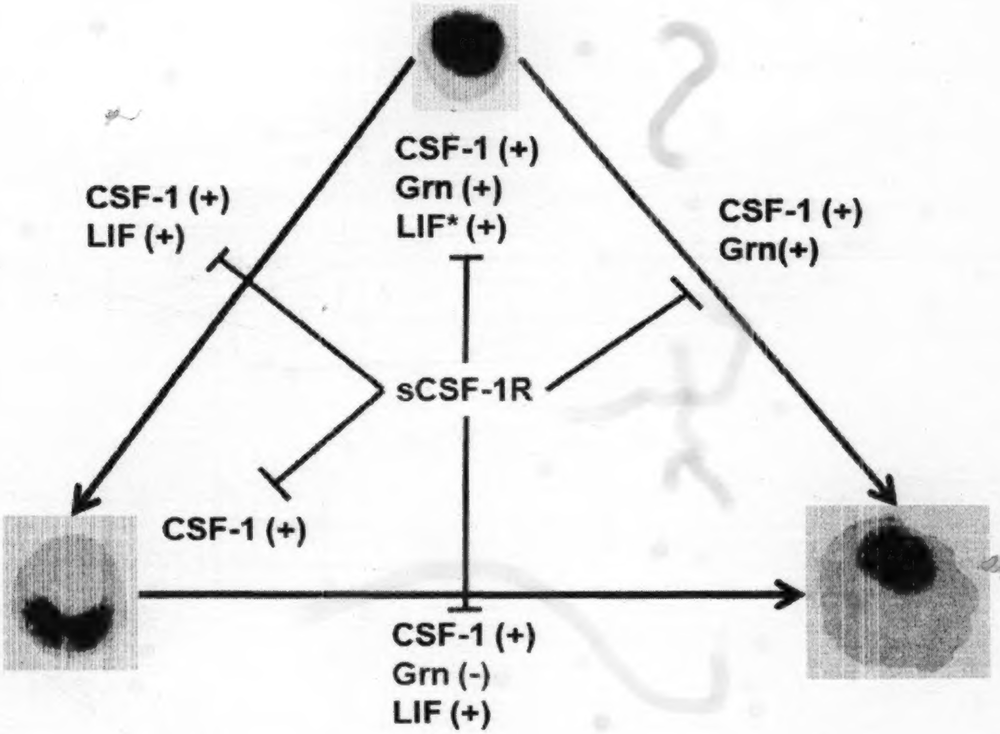

Figure 8.1 Model summarizing the effects of growth factors on myelopoiesis in the goldfish. Model summarizing the influences of goldfish CSF-1, granulin and LIF on progenitor cell (top), monocyte (bottom left) and macrophage (bottom right) development. Growth factors placed near each cell type are involved with the proliferation of that cell type, and growth factors placed near lines transitioning from one cell type to another are involved in the differentiation process. Growth factors depicted in blue with a (+) promote a process whereas those in red with a (-) inhibit or block a process from occurring. The * beside LIF indicates that this process requires other factors in combination with LIF in order to result in the effect indicated.

8.1 Future Directions

In this thesis, I describe experiments I performed to characterize the growth factors CSF-1, granulin and LIF in the goldfish. Much of my work has focused on elucidating the impact of each growth factor on the proliferation and differentiation of cells involved in myelopoiesis. The primary purpose of this work was to begin to outline a model for goldfish macrophage development by characterizing the individual effects of growth factors, CSF-1, granulin and LIF, involved in this process. Although the characterization of these three growth factors represents a significant leap forward in our understanding of fish myelopoiesis, much work remains to be done to identify other myelopoietic growth factors and beginning to understand how all of these growth factors work in unison to drive developmental changes. I believe that there are at least four essential studies that should be undertaken in order to fully understand macrophage development in the goldfish. (A) Functional assessment of recombinant and native growth factors *in vitro* to better understand their individual roles in myelopoiesis of the goldfish. (B) Examination of the interactions among the growth factors with specific reference to regulation of proliferation and differentiation of goldfish macrophages. These experiments are critical for understanding how cells respond to more than one molecule at a time, a situation that is a feature of hematopoietic microenvironments. (C) Development of stable long-term macrophage cell lines to further reduce the variability of the PKM goldfish macrophage system. This will allow for much more accurate studies to be performed on the biology of fish macrophages. (D) Identification and expansion of the database of distinct cell developmental stage markers. This will allow for much more in depth studies on myelopoiesis both *in vitro* and *in vivo*.

(A) Functional comparison of recombinant and native macrophage growth factors:

The majority of the work presented in this thesis was done using recombinant proteins produced by various expression systems. These recombinant proteins were very useful for initial assessment of the functions of goldfish macrophage growth factors. However, to fully understand how these growth factors work, experiments must be undertaken to identify and isolate the native proteins. Based on the evidence gathered using mammalian model systems, it is likely that the functions of native and recombinant proteins will be similar. However, the biological levels required to stimulate a specific response, the growth factor-receptor interactions and the exact functional forms of each growth factor can only be fully addressed using native proteins.

Colony stimulating factor-1. CSF-1 appears to function much like its mammalian counterpart. Identification of the native goldfish CSF-1 using the polyclonal antibody generated against the recombinant peptide will be critical for further assessment of CSF-1 function in fish. One major difference between the CSF-1s of lower and higher vertebrates is the pool of distinct alternatively spliced variants which has implications regarding the CSF-1 function. In preliminary experiments, I found that supernatants of goldfish CCL-71 cell cultures contain significant growth-inducing activity for PKM, I have also shown that the growth-inducing activity in CCL-71 supernatants was blocked by the addition of sCSF-1R, suggesting that CSF-1 is primarily responsible for this activity. Therefore, it will be possible to purify the native CSF-1 from these cell culture supernatants using immune-affinity chromatography. In addition, this system will allow for the identification and characterization of different native protein forms present in CCL-71 supernatants, as well as CCM from actively growing PKM cultures.

Granulin. The induction of proliferative response of the progenitor cell pool *in vitro* by the recombinant goldfish granulin demonstrated in this thesis is very interesting and I believe that further characterization of its biological role in myelopoiesis is warranted. This will be challenging because, over the years, researchers have attempted to find the granulin receptor(s) using a variety of techniques. The identification of the granulin receptor(s) is of central importance for the full functional analysis of this growth factor of macrophages. Once the granulin receptor is identified, the biological differences between the short hematopoietic granulin identified in this thesis and the longer progranulins present in mammals and zebrafish can be examined. Furthermore, the identification of specific cell types in the progenitor cell pool that are responsive to granulin stimulation may provide information that will allow researchers interested in teleost biology to more easily establish long-term cell lines in fish.

Leukemia inhibitory factor and its receptor. Goldfish LIF and LIFR have a great deal of potential for fish developmental research. Mammalian LIF is extremely important in stem cell research and the potential to use fish for stem cell research is very appealing given the societal concerns of using mammalian stem cells. However, before fish model systems can be used for stem cell research, a variety of stem cell markers that have been identified in mammals need to be characterized in fish.

The determination of the evolutionary placement of LIF and LIFR may potentially be an interesting project. As indicated earlier, the mammalian IL-6 cytokine family members have significant functional redundancy, in part due to their communal utilization of gp130 receptor. Thus, the understanding of the evolutionary origin of this family of cytokines, and in particular the relationship between LIF and OSM, may provide clues with regards to the generation of redundancy of immune responses in general. I believe that based on my thesis work, the goldfish model system is ideal for exploring the possibility that LIF and OSM originated as 'M17' molecule, which I have identified as goldfish LIF, suggesting that LIF may be ancestral to OSM. Although the demonstration of these types of relationships will be difficult, further functional analysis of goldfish LIF may provide information that will eventually shed light on the generation of redundancies that are a feature of immune responses.

(B) Examination of the interactions among the growth factors with specific reference to regulation of proliferation and differentiation of goldfish macrophages:

Defining the effects of individual growth factors on PKM development is of central importance for understanding their role in myelopoiesis of teleosts. As discussed earlier, during myelopoiesis, the cells are simultaneously exposed to a plethora of different growth factors in hematopoietic microenvironments, all of which influence cell development. Therefore, it is important not only to examine the effects of individual growth factors on developing cells but also their communal effects during myelopoiesis. The availability of the recombinant growth factors for goldfish macrophages characterized in this thesis allows for the examination of their combinatory effects on developing goldfish macrophages *in vitro* and *in vivo*. The overlapping functions of CSF-1, LIF and granulin in goldfish suggest that they would all be present and influencing monocyte/macrophage development and function under certain conditions. Experiments in which these growth factors were combined together would provide insight into how the complex regulation of monocyte/macrophage development occurs in the goldfish.

(C) Development of stable long-term fish macrophage cell lines:

The importance of stable, biologically predictable, long-term cell lines in mammalian immunological and hematopoietic research cannot be understated. The lack of cell lines and long-term homogenous cultured cells is an impediment for the identification and characterization of precise mechanisms that control fish hematopoiesis and fish immune responses. Having goldfish cell lines that represent distinct developing macrophage subpopulations (progenitor cells, monocytes and mature macrophages) would enhance our ability to identify developmental stage-specific cell markers and study the differentiation mechanisms during myelopoiesis. I have shown in this thesis that addition of rgCSF-1 to PKM cultures resulted in a more homogenous macrophage population compared to the cultures that were not treated with rgCSF-1. Although a few monocytes/macrophages cell lines have been established from fish [461, 463, 577], I believe studies aimed at establishing long-term monocyte/macrophage cell lines using rgCSF-1 will provide a procedural map for development of monocyte/macrophage cell lines not only in goldfish but also other teleosts.

(D) Identification and expansion of the database of distinct cell developmental stage markers.

Researchers using zebrafish model have generated a significant database of markers for different developmental cell stages and types. Many of these markers are highly conserved transcription factors that have been used with great success in transgenic zebrafish to detect, isolate and study specific cells populations [31, 33, 36]. Unfortunately, the generation of transgenic goldfish is a difficult undertaking making many of the markers identified for zebrafish non compatible for use in a goldfish model system. Therefore, there is a need for the identification and characterization of cell surface markers for developing goldfish cells. These cell markers can be used to isolate and functionally characterize stem cell and/or effector cell populations as demonstrated by research using mammalian systems.

In summary, the information that will be generated by conducting the studies I outlined above, would further enhance our knowledge about myelopoiesis in the goldfish and may provide a path for the examination of the myelopoieis from an evolutionary perspective not only in teleosts but also other metazoa.

Chapter 9

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